AWARD NUBMER:	W81XWH-11-1-0211
Title:	"The Role of Novel Substituted Diindolyl Methane Analogues in the Treatment of Triple-Negative and ErbB2-Positive Breast Cancer"
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REPORT DATE:	MAY 2015
TYPE OF REPORT:	ANNUAL
PREPARED FOR:	U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012
DISTRIBUTION STATEMENT: Approved for Public Release; Distribution Unlimited	

REPORT DOCUMENTATION PAGE		*Form Approved* *OMB No. 0704-0188*
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1. REPORT DATE **May 2015**	**2. REPORT TYPE** **Annual**	**3. DATES COVERED** **1May2014 - 30Apr2015**
4. TITLE AND SUBTITLE "The Role of Novel Substituted Diindolyl Methane Analogues in the Treatment of Triple-Negative and ErbB2-Positive Breast Cancer"		**5a. CONTRACT NUMBER** **5b. GRANT NUMBER** **W81XWH-11-1-0211** **5c. PROGRAM ELEMENT**
6. AUTHOR(S) **Dr. Mandip S. Sachdeva** email: mandip.sachdeva@famu.edu		**5d. PROJECT NUMBER** **5e. TASK NUMBER** **5f. WORK UNIT NUMBER**
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **Florida A&M University** Tallahassee, FL 32307		**8. PERFORMING ORGANIZATION REPORT**
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012		**10. SPONSOR/MONITOR'S ACRONYM(S)** **11. SPONSOR/MONITOR'S NUMBER(S)**
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited		
13. SUPPLEMENTARY NOTES		
14. ABSTRACT The mission of the proposed HBCU/MI Breast Cancer Partnership Training Award at Florida A & M University (FAMU) is to develop a rich intellectual environment that will promote and strengthen the research capabilities of FAMU investigators in the area of breast cancer research. The objectives this proposal are (1) to provide mentorship and training to FAMU researchers in breast cancer research area to enhance the research expertise and competitive ability; (2) to train FAMU investigators through a well defined research project investigating the anticancer potential of C-DIM analogs in treatment of breast cancer; (3) to develop FAMU investigators grantsmanship skills by submitting extramural grants for independent funding; and (4) to create awareness among FAMU researchers and African American Community about breast cancer biology and therapy. The outcome of this proposal will lead to novel oral therapeutic strategies for treatment of triple negative breast cancer (TNBC) and ErbB2-positive breast cancer) EPBC and also result in publications in highly ranked journals. This approach will result in establishment of a successful and independently funded breast cancer research program at FAMU. Our ultimate goal is to become an independent research and training program of excellence for minority investigators		

15. SUBJECT TERMS **Triple negative breast cancers**					
16. SECURITY CLASSIFICATION OF:			**17. LIMITATION**	**18. NUMBER**	**19a. NAME OF RESPONSIBLE PERSON** USAMRMC
a. REPORT U	**b. ABSTRACT** U	**c. THIS PAGE** U	UU	57	**19b. TELEPHONE NUMBER** *(include area code)*

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2. Section I: A brief introduction covering the purpose and scope of the research effort

Breast cancer is the second leading cause of cancer-related deaths in the United States with 40,000 deaths and 200,000 new cases diagnosed annually. Approximately 15%-20% of patients are diagnosed with TNBC, which do not express estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2). It is a particularly lethal subtype of breast cancer with a 5-year survival rate as low as 40%. TNBC is highly malignant tumor with significantly greater risk for recurrence and shortened survival compared with other types of breast cancer. The life expectancy after detection of visceral metastasis in TNBC patients is estimated as 3 to 22 months. The over-expression of ErbB2 (HER2) occurs in approximately 20–25% of all breast tumors and the outcome of current therapies for ErbB2-positive breast cancers (EPBC) remains unsatisfactory due to short intervals to recurrence, short overall survival, resistance and toxic side effects. African American women are 34% more likely to die from breast cancer than white women. The TNBC and EPBC are high risk breast cancers and the choice of orally available chemotherapeutic agents is limited. Hormonal therapy (ER modulators) and HER antibody based therapy are far safer than cytotoxic drug based regimens. But triple negative breast cancers are not responsive to hormonal or HER targeting therapy. Given the challenge in treating TNBC and EPBC and its inherent poor prognosis, the use of novel orally active C-DIM analogues will have major clinical implications for the treatment of breast cancer. Orally administered Diindolyl methane (DIM) analogue DIM-C-pPhC6H5 alone showed potent anticancer activity and also exhibited additive to synergistic anticancer activity in combination with docetaxel (doc). Based on this success, our ultimate objective is to design, synthesize and evaluate the novel orally effective DIM analogues to treat TNBC and EPBC by establishing the breast cancer research program with the support of proposed HBCU/MI Partnership Training Award. The proposed studies will synthesize C-DIM analogues and investigate the structure-dependent anticancer activities in TNBC and EPBC using both cell and laboratory animal models. Our hypothesis is that the novel C-DIM analogues will show potent anticancer activity against TNBC and EPBC and the use of C-DIM analogues could be a novel approach for the treatment of breast cancer.

3. Section II: Research Accomplishments

Synthesis of various C-DIM analogues, in vitro cytotoxicity, pharmacokinetic in rats and higher animals in vivo anticancer efficacy studies and western blot to analyze various tumor markers were summarized in the previous reports (December 2011, May 2012, April 2013, May 2014). Compounds, C-DIM-10 and C-DIM-14 were found to have superior anticancer activity against TNBC (MDA-MB-468, MDA-MB-231 and MDA-MB-453) and EPBC (BT474 and SKBR3) cells in comparison to other DIM analogues. Both the compounds were founds to have poor bioavailability due to their poor solubility. Therefore, DIM-10 loaded nanostructured lipid carrier (NLC) was developed to improve their oral bioavailability. Pharmacokinetic of free drug and DIM NLC in dog were described in our last report of May 2014 while Pharmacokinetic and anticancer efficacy of DIM loaded nanocarrier in TNBC tumor bearing mice was described in our previous report of 2012 and 2013.

In current year, we have investigated the cytotoxic disparity of DIM in Caucasian and African American TNBC cell lines. However, we did not observed any difference in anticancer activity of DIM towards cell lines from different origin and currently we are looking for more tumor specimens to look at the area of disparity in response.

The novel role of DIM as an orphan nuclear receptor 4A1 (NR4A1) antagonist was explored in TNBC. NR4A1 is overexpressed in mammary tumors and transfection of MDA-MB-231 breast cancer cells with siNR4A1 decreased cell proliferation and induced apoptosis. NR4A1 binds and inactivates p53 and knockdown of NR4A1 or treatment of p53 wild-type lung cancer cells with an NR4A1 antagonist or transfection with siNR4A1 results in activation of p53 and induction of sestrin 2 which activates AMPKα and inhibits the mTOR pathway (Figure 1).

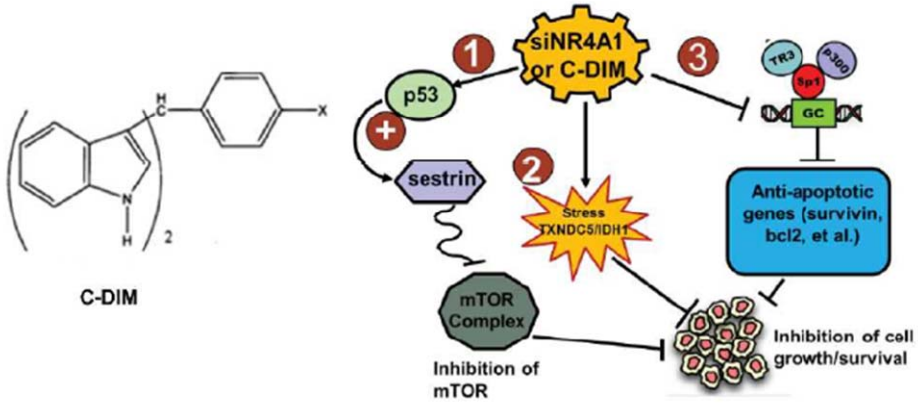

Figure 1. NR4A1-regulated pathways and effects of NR4A1 knockdown on breast cancer cell proliferation. NR4A1-regulated pathways/genes that can be targeted by C-DIM/NR4A1 antagonists.

Both the DIM derivatives showed significant reduction in tumor weight and volume in MDA-MB-231 orthotropic tumor bearing mice (Figure 2). Effects of C-DIM/NR4A1 antagonists were comparable to that observed after NR4A1 knockdown. The C-DIM compounds with a p-carboxymethylpenyl group (DIM-C-pPhCO2Me) and cyano substitutent (DIM-C-pPhCN) have been identified as NR4A1 antagonists. After knockdown of NR4A1 in these cells, treatment with DIM-C-pPhCO2Me resulted in only minimal growth inhibition confirming a role for NR4A1 in mediating the growth inhibitory effects of DIM-C-pPhCO2Me (Figure 3A). Treatment of MDA-MB-231 cells with DIM-C-pPhCO2Me also increased ROS after 12 and 24 hr (Figure 3B) and this was also accompanied by decreased expression of TXNDC5 and IDH1 and induction of markers of ER stress (p-PERK, ATF4, CHOP and XBP-1s). Treatment of the cells with DIM-C-pPhCO2Me for 24 hr induced cleavage (activation) of caspases 7 and 8 and PARP (Figure 4A) and enhanced annexin V staining in MCF-7 (Figure 3C) and similar results were observed for the p-cyanophenyl compound (DIM-C-pPhCN). The NR4A1 antagonist DIM-C-pPhCN also inhibited the mTOR pathway. siNR4A1 or treatment with DIM-C-pPhCO_2Me decreased expression of survivin, bcl2 and EGFR in MDA-MB-231. Reduction in the expression of same markers was observed in *in vivo* anticancer studies in MDA-MB-231 xenograft solid tumor bearing athymic nude mice (Figure 4B and 4C). DIM-C-pPhCO_2Me and siNR4A1 also induced sestrin 2 and inhibited mTOR in p53 mutant SKBR3 and MDA-MB-231 cells and the mechanisms of this response are currently being investigated.

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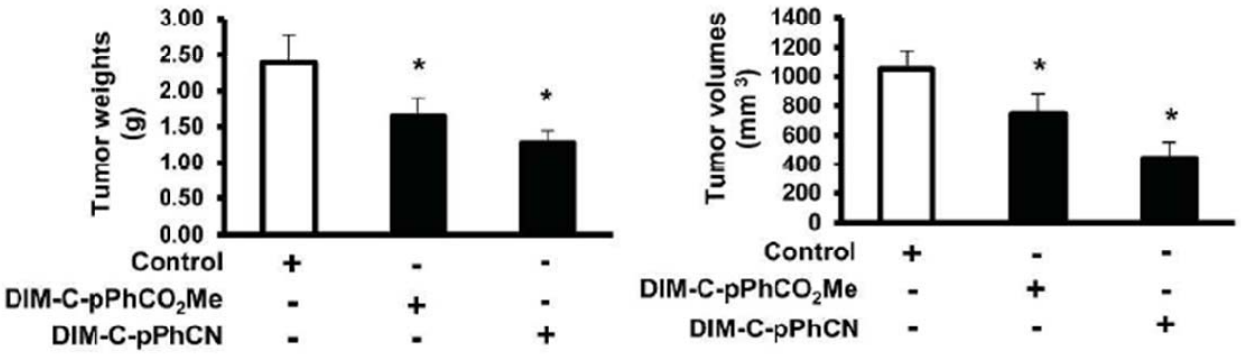

Figure 2. Athymic nude mice bearing MDA-MB-231 cells (orthotopic) were administered corn oil (control), DIM-C-pPhCO$_2$Me or DIM-C-pPhCN (50 mg/kg d) by oral gavage for 28 days, and effects on tumor growth and weight were determined (* significantly decreased; $p<0.01$).

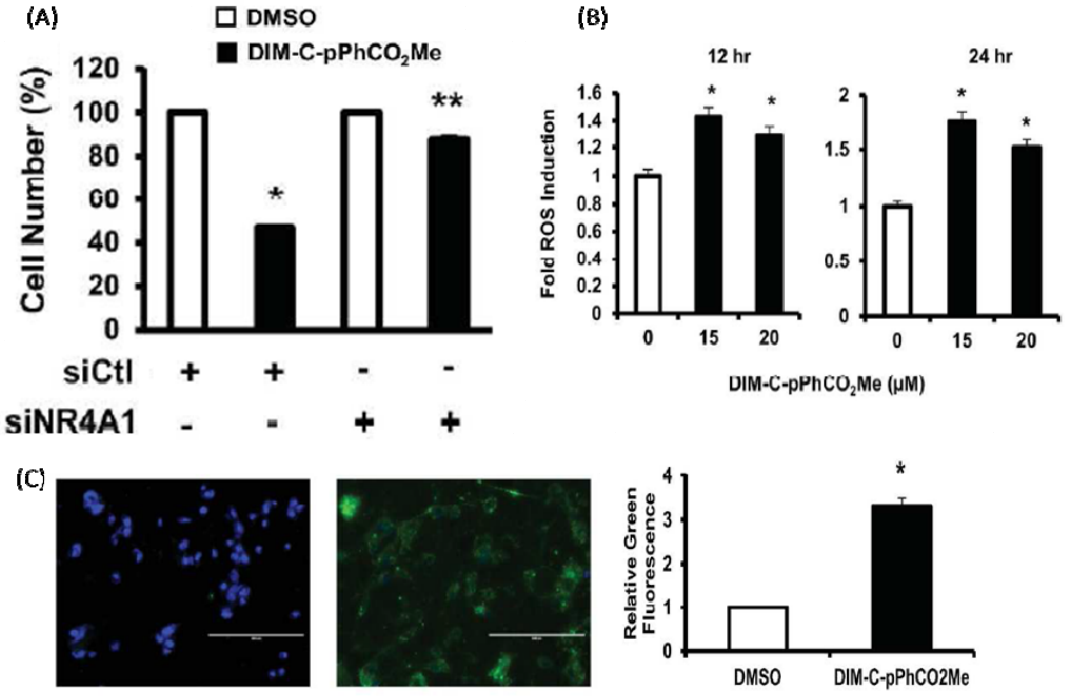

Figure 3. (A) NR4A1-regulated pathways and effects of NR4A1 knockdown on breast cancer cell proliferation. Cells were transfected with siCtl (non-specific oligonucleotide) or siNR4A1 and then treated with 20 μM DIM-C-pPhCO$_2$Me for 24 hr and the number of cells were then counted. (B) DIM-C-pPhCO$_2$Me induces ROS and stress. Cells were treated with DIM-C-pPhCO$_2$Me and ROS was determined after 12 or 24 hr. (C) DIM-C-pPhCO$_2$Me induces apoptosis in cells were treated with DIM-C-pPhCO$_2$Me for 24 hr. Annexin V staining

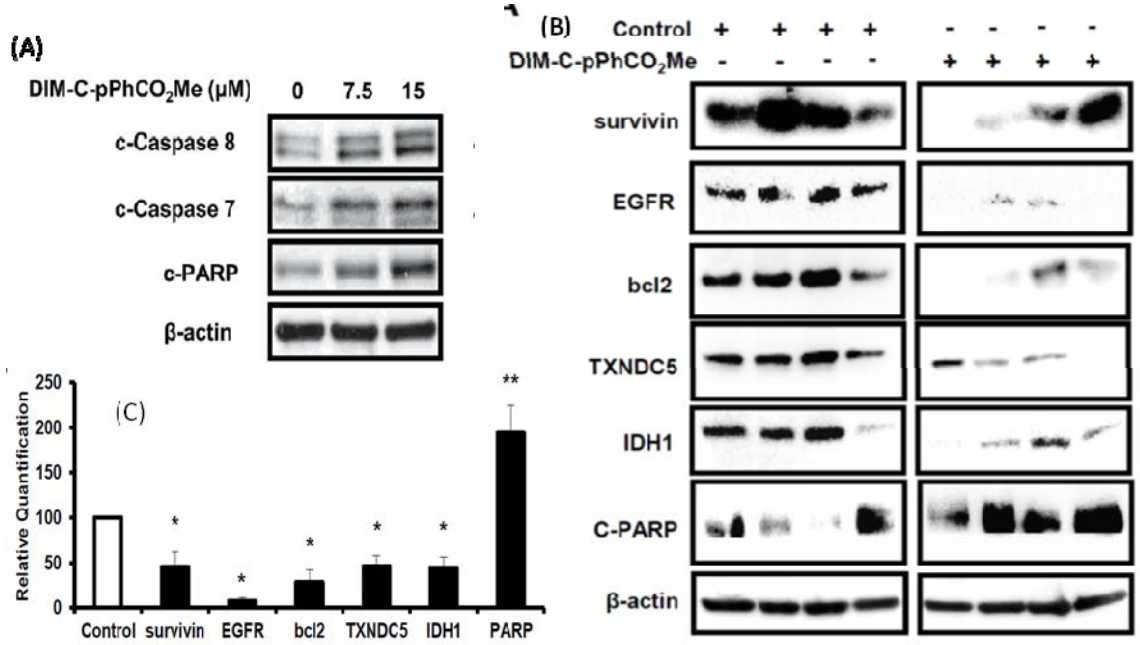

Figure 4. (A) DIM-C-pPhCO$_2$Me induces apoptosis in cells were treated with DIM-C-pPhCO$_2$Me for 24 hr. Whole cell lysates were analyzed by western blots (B) Effects of NR4A1 antagonists on selected gene product expression in tumors. Orthotopic tumors in athymic nude mice bearing MDA-MB-231 cells were treated with DIM-C-pPhCO2Me (50 mg/kg/d) or vehicle (control) for 28 days. Lysates from tumors from control and DIM-C-pPhCO2Me-treated mice were analyzed by western blots and (C) bands were quantitated and normalized relative to β-actin.

Collaboration and training:

a) Dr. Sachdeva and Dr. Stephen Safe are in touch on a weekly basis to plan the experiments. Further several discussions have led to understanding novel mechanism of action of C-DIM compounds and explore it for other cancers. Further, Dr. Musa who was involved in the synthesis of compounds has prepared some compounds which have been further evaluated.

b) Ms. Valerie Marcellus and Ms. Jane Hong, undergraduate students have been trained and will be working in Dr Sachdeva's lab to study the in vitro anticancer studies.

c) Current post doc under this project Dr. Ketan Patel been actively involved in the exploring DIM analogues for their pharmacokinetic evaluation and in formulating nanoparticles to enhance their bioavailability.

d) PI has been conducting several journal clubs in his lab to discuss the latest developments in the field of cancer chemotherapy and nanotechnology. The aim of these journal clubs is to stimulate discussions and new ideas in breast cancer.

4. **Section III: Problem Areas**
None

Section IV: A description of work to be performed during the next reporting period.
For the next one year, the following experiments and activities are planned:

a. To conduct experiments with transgenic mice and also patient derived tumor models (PDX models). These experiments will demonstrate the translational outcome of this research.
b. To prepare nanoparticles containing DIM compounds which are surface modified with various peptides specific for targets on tumor cells. These nanoparticles will be ten further studied in vitro and in vivo.
c. To submit R01 proposal in the area of breast cancer

Section V: Administrative Comments (Optional) - Description of proposed site visits and participation in technical meetings, journal manuscripts in preparation, coordination with other organizations conducting related work, etc.

Presentations:

1. Jaganmohan Somagoni, Chandraiah Godugu, Ravi Doddapaneni, Stephen H Safe and **Mandip Singh**. Treatment of pulmonary arterial hypertension by the use of a novel PPAR gamma agonist, diindolylmethane in a nanoparticle formulation. American Association of Pharmaceutical Science (AAPS) Annual Meeting & Exposition 2014, San Diego, CA.

Publications:

1. Erick Hedrick, Syng-Lee, Ravi Doddapeneni, **Mandip Singh** and Stephen Safe. Nuclear Receptor 4A1 (NR4A1) as a Drug Target for Breast Cancer Chemotherapy. Accepted in Endocrine Related cancer 2015. **Both Mandip Singh and Stephen Safe are corresponding authors in this paper.**

2. Safe S[1], Jin UH, Morpurgo B, Abudayyeh A, **Singh M**, Tjalkens RB. Nuclear receptor 4A (NR4A) family - orphans no more. J Steroid Biochem Mol Biol. 2015 Apr 23. pii: S0960-0760(15)00113-2. doi: 10.1016/j.jsbmb.2015.04.016. [Epub ahead of print].

Manuscript under preparation:

1. Reeder, A, **Singh M**, Patel K, Doddapeni R, Hedric E, Safe S. Nuclear receptor4A1(NR4A1) as a drug target for treating rhabdysarcoma cells. Manuscript under preparation.

1. References

2. Appendices

Journal of Steroid Biochemistry & Molecular Biology xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry & Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Nuclear receptor 4A (NR4A) family – orphans no more

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ARTICLE INFO

Article history:
Received 15 January 2015
Received in revised form 26 March 2015
Accepted 21 April 2015
Available online xxx

Keywords:
NR4A
Multiple functions
Nuclear receptors
Novel ligands

ABSTRACT

The orphan nuclear receptors NR4A1, NR4A2 and NR4A3 are immediate early genes induced by multiple stressors, and the NR4A receptors play an important role in maintaining cellular homeostasis and disease. There is increasing evidence for the role of these receptors in metabolic, cardiovascular and neurological functions and also in inflammation and inflammatory diseases and in immune functions and cancer. Despite the similarities of NR4A1, NR4A2 and NR4A3 and their interactions with common *cis*-genomic elements, they exhibit unique activities and cell-/tissue-specific functions. Although endogenous ligands for NR4A receptors have not been identified, there is increasing evidence that structurally-diverse synthetic molecules can directly interact with the ligand binding domain of NR4A1 and act as agonists or antagonists, and ligands for NR4A2 and NR4A3 have also been identified. Since NR4A receptors are key factors in multiple diseases, there are opportunities for the future development of NR4A ligands for clinical applications in treating multiple health problems including metabolic, neurologic and cardiovascular diseases, other inflammatory conditions, and cancer.

1. Introduction

The orphan nuclear receptor (NR) family has been characterized as a collection of nuclear receptors which share many structural domain similarities with other NRs; however, their endogenous ligands are unknown [1]. These receptors include NR0B1 (adrenal hypoplasia congenita critical region on chromosome X gene), NR0B2 (small heterodimer partner), NR1D1/2 (Rev-Erbαβ), NR2C1 (testicular receptor 2), NR2C2 (testicular receptor 2), NR2E1 (tailless), NR2E3 (photoreceptor-specific NR [PNR]), NR2F1 chicken ovalbumin upstream promoter transcription factor 1 (COUP-TFI), NR2F2 (COUP-TFII), NR2F6 (verbA-related protein), NR4A1 (Nur77), NR4A2 (Nurr1), NR4A3 (Nor1), and NR6A1 (GCNF).

In contrast to the other NRs, the orphan NR0B1 (DAX-1) and NR0B2 (SHP) receptors do not express a DNA binding domain (DBD) and primarily function as nuclear cofactors that influence gene expression through protein–protein interactions [2–4]. The three NR4A receptors have significant structural similarities in their ligand binding domains (LBDs) and DNA BDs, whereas their N-terminal (A/B) domains containing activation function 1 (AF1) are highly divergent [5–8]. NR4A receptors were initially defined as nerve growth factor-induced-β (NGFI-β) receptors that bind as monomers to an NGFI-β response element (NBRE:AAAGGTCA) [8–12]. NR4A receptors also bind as a homo- or hetero-dimer to a nur-responsive element (NuRE:TGATATTACCTCCAAATGCCA) which has been characterized from the pro-opiomelanocortin gene promoter [13,14]. Both NR4A1 and NR4A2 can also bind as heterodimers with the retinoid X receptor (RXR) to a DR5 motif [15,16]. These receptor-DNA interactions are characteristic of all NRs (except NR0B1 and NR0B2) and there is also evidence that NR4A1 regulates gene expression through interactions with the specificity protein 1 (Sp1) transcription factor bound to its cognate GC-rich motif [17–19]. NR4A1 acts as a cofactor (along with p300) of Sp1, and many other NRs bind Sp1 and are integral cofactors for expression of Sp1-regulated genes [19–28].

The initial discovery of NR4A receptors was linked to their rapid induction by multiple stimuli in various tissues/cells and organs and these responses play a role in coping with both exogenous and endogenous stressors and the tissue-specific expression and induction of NR4A receptors contributes to their specificity

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http://dx.doi.org/10.1016/j.jsbmb.2015.04.016
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(reviewed in [29,30]). For example, NR4A receptors are induced by nerve growth factors in neuronal cells and by apoptosis-inducing agents in cancer cell lines [31–37]. In contrast, extensive studies with NR4A1 demonstrate that this receptor is not only induced by diverse anti-apoptotic agents but is also highly expressed in solid tumors and exhibits pro-oncogenic activity. Over the past decade, several timely and informative reviews on NR4A receptors have been published [29,30,38–42] and therefore this paper will primarily focus on more recent advances in the field.

2. NR4A1 in cellular homeostasis and diseases

Individual and combined knockouts of NR4A1, NR4A2 and NR4A3 in mice have been described and extensively investigated to demonstrate the function of these receptors in maintaining cellular homeostasis and their role in disease. Thus, contributions of NR4A1 in metabolic disease, inflammation, atherosclerosis and other responses will be discussed in subsequent sub-sections of this review. One of the earliest functions identified for NR4A1 was its induction in T-cell hybridomas or thymocytes undergoing apoptosis [43,44]. Surprisingly, T-cell receptor-mediated apoptosis in NR4A1 knockout mice was not defective and other apoptosis inducers were also functional in these mice [45]. NR4A1 also modulates adrenocortical function by regulation of CYP21 expression; however, in NR4A1 knockout mice the function of the hypothalamic pituitary axis was intact and it was concluded that other factors expressed in these mice compensated for loss of NR4A1 [46,47]. However, the loss of NR4A1 in mice has dramatic effects on inflammatory, immune, metabolic and neurological functions and these will be discussed under the proceeding subsections. The direct effects of NR4A1 loss in mice were more pronounced in some double knockout mice containing the loss of another NR4A gene. For example, the loss of both NR4A1 and NR4A3 in mice led to the rapid development of lethal acute myeloid leukemia (AML) in mice indicating tumor suppressor-like activity for these receptors [48]. Using a similar approach, it was shown that a decreased dose of NR4A1 and NR4A3 (*e.g.*, NR4A1$^{+/-}$/NR4A3$^{-/-}$ and NR4A1$^{-/-}$/NR4A3$^{+/-}$) resulted in a condition resembling a mixed myelodysplastic/myoproliferative neoplasm [49]. It was also recently reported that loss of NR4A1, NR4A2 and NR4A3 in T-cells blocked development of Treg-cells and resulted autoimmune diseases in multiple organs [50]. Thus, the future development of tissue-specific knockout of one or more NR4A receptors in mice will be important for understanding the underlying functions of these receptors.

2.1. NR4A1 and metabolic diseases

Pearen and Muscat have reviewed the roles of NR4A1 and other NR4A genes in metabolic diseases [30] and have summarized the diverse stimuli associated with metabolic function that induce expression of NR4A receptors and their role in glycogen metabolism in skeletal muscle has been reviewed [51]. Several recent reports have expanded on the role of NR4A1 in obesity and type2 diabetes and the potential for using NR4A1 ligands for treating this disease which has been increasing dramatically in western industrialized countries. NR4A1, NR4A2 and NR4A3 are highly upregulated in obese individuals and significantly decrease after fat loss [52]. NR4A1, NR4A2 and NR4A3 are rapidly induced by cAMP in mouse hepatocytes and by glucagon in mouse liver and overexpression of NR4A1 induced gluconeogenic gene expression [53]. In mice injected with an adenoviral-NR4A1 construct there was an increase in blood and hepatic glucose levels, whereas a dominant negative adenoviral-NR4A1-M1 construct decreased blood glucose levels and other parameters consistent with a diabetic-like condition [53]. In contrast the protective effects of NR4A1 knockdown in normal mice is not observed in NR4A1$^{-/-}$ mice maintained on a high fat diet since these animals exhibit increased insulin resistance and hepatic steatosis [54]. This study also demonstrated that loss of NR4A1 increases insulin-resistance suggesting that NR4A1 expression in muscle and other tissue may influence whole body glucose metabolism and metabolic disease [54]. In diabetic db/db mice expressing NR4A1, blood glucose levels were higher than in the db/db/NR4A1$^{-/-}$ mice, whereas levels were similar in normal mice and high fat diet plus streptozotocin (STZ) mice which represent a non-genetic model for obesity and T2DM [55]. The rationale for the differences in NR4A1 function in these mouse models requires further investigation.

Wu and coworkers have been investigating the identification and effects of NR4A1 ligands on metabolic disease and have identified cytosporone B (CsnB) and related analogs and ethyl [2,3,4-trimethoxy-6-(*i*-octanoyl)phenyl]acetate (TMPA) as compounds that bound the ligand binding domain (LBD) of NR4A1 (Fig. 1) [34,55,56]. CsnB was characterized as an NR4A1 agonist that increased blood glucose levels and induced hepatic gluconeogenesis in C57BL/6 mice [56]. TMPA also interacted with the LBD or NR4A1 but in contrast to CsnB, TMPA decreased blood glucose in db/db mice and had lower levels of insulin and the effects were not observed in db/db/NR4A1$^{-/-}$ mice [55]. Moreover, in the non-genetic high fat diet/STZ-treated mice TMPA also decreased blood glucose levels and the effects were not observed in these mice after loss of NR4A1. In addition, TMPA also inhibited hepatic gluconeogenesis in db/db mice as evidenced by increased phosphorylation of AMPKα and repression of glucose-6-phosphatase (G6pc) and phosphoenolpyruvate carboxykinase (Pepck) gene expression which was not observed in db/db mice crossed with NR4A1$^{-/-}$ mice. NR4A1-dependent activation of hepatic gluconeogenesis has been reported in several studies [53,56,57] and using TMPA (Fig. 1) as an NR4A1 antagonist, an interesting pathway has been uncovered (Fig. 1B) [55]. High levels of gluconeogenesis are associated, in part, with constitutive inactivation of AMPKα due to the inactivation of liver kinase B1 (LKB1) which is sequestered by NR4A1 in the nucleus. Inactivation of NR4A1 in cells treated with TMPA results in nuclear export of free LKB1 which in turn activates (phosphorylates) AMPKα resulting in the inhibition of gluconeogenesis [55].

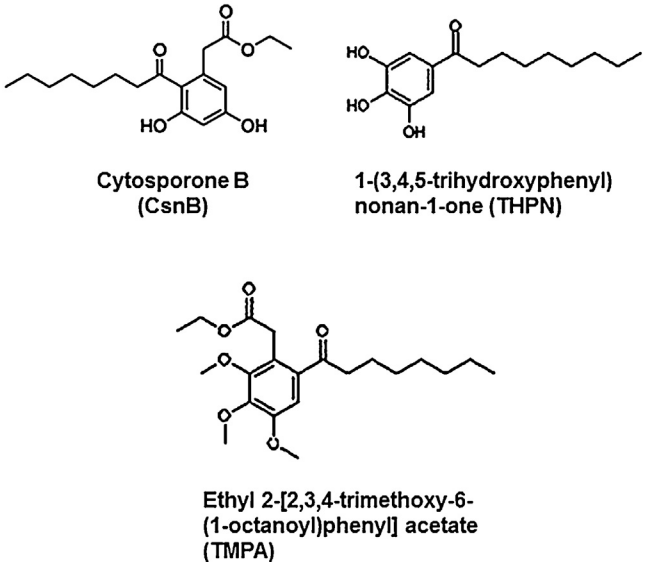

Fig. 1. Ligands that bind NR4A1. Studies in the Wu laboratory have identified a series of polyhydroxyaromatic compounds containing medium chain alkylketone groups that bind NR4A1 and act as agonists and antagonists (CsnB, THPN and TMPA) (34, 55, 56 and 127).

Please cite this article in press as: S. Safe, et al., Nuclear receptor 4A (NR4A) family – orphans no more, J. Steroid Biochem. Mol. Biol. (2015), http://dx.doi.org/10.1016/j.jsbmb.2015.04.016

Thus, it is apparent that NR4A1 plays an important role in metabolic disease and T2DM and is a potential target for treatment of metabolic diseases and their complications.

2.2. NR4A1 and cardiovascular disease

Since cardiovascular disease is associated with chronic inflammation, it is not surprising that NR4A receptors play a role in this disease (reviewed in [58–60]). NR4A1 is expressed and functional in many of the cell subtypes that contribute to the damage of arterial vessel cell walls and this includes vascular smooth muscle cells, endothelial cells, invading macrophages and monocytes. De Vries and coworkers first detected NR4A1 as a gene induced in human smooth muscle cells treated with growth factors and cytokines [61] and also in atherosclerotic lesions in mouse models [62–65]. Perturbation of smooth muscle cells increases NR4A1 expression, and results of knockdown or overexpression experiments suggest that this receptor inhibits proliferation [63,64]. Balloon-injury induced neointimal hyperplasia in rat carotid arteries was inhibited after treatment with the antioxidant α-lipoic acid which also induced formation of cytoplasmic NR4A1 [66]. The protective effects of α-lipoic acid were decreased after NR4A1 knockdown *in vivo*. In vascular smooth muscle cells in culture, NR4A1 was important for α-lipoic acid-induced apoptosis, suggesting that the cytosolic NR4A1 is critical for induction of apoptosis and this is consistent with studies in cancer cells and tumors [41]. Similar results were observed in neonatal heart cells cultured under conditions resembling a high fat diet where reactive oxygen species (ROS) induced apoptosis and this was accompanied by increased cytosolic NR4A1 expression and interactions with the mitochondria as observed in some cancer cell lines [41].

NR4A1 is also induced by multiple factors in endothelial cells and plays a role in endothelial cell proliferation and angiogenesis [67–69]. A recent study reported that both histamine and serotonin are pro-angiogenic factors in endothelial cells and *in vivo* and these effects are dependent on the histamine and serotonin receptors and NR4A1 but are independent of vascular endothelial growth factor (VEGF). These responses are also transitory since after an extended period (10 day), the angiogenesis inhibitor thrombospondin 1 was induced by serotonin and histamine and this response was NR4A1-independent [70]. The role of NR4A1 in inflammation and macrophages will be discussed separately; however, macrophages in areas of plaque formation express NR4A1 [70,71]. Moreover, in both cell models and ApoE$^{-/-}$ mice maintained on a high fat/cholesterol diet, increased expression of NR4A1 or activation of the receptor by CsnB decreased macrophage derived foam cells and decreased atherosclerotic plaque formation [72]. This was also accompanied by decreased expression of inflammatory and adhesion genes and decreased hepatic lipid deposition and intestinal absorption of lipids, whereas the opposite effects were observed after NR4A1 knockdown. These results were consistent with transgenic animal studies showing that expression of NR4A1 results in inhibition of macrophage accumulation and matrix metalloproteinase levels in mouse models [62]. Complementary results [73] were also observed in ApoE$^{-/-}$/NR4A1$^{-/-}$ mice that exhibited increased atherosclerosis after 11 weeks on a western diet, and the loss of NR4A1 enhanced atherosclerosis, enhanced toll-like receptor signaling and pro-inflammatory macrophages. The importance of NR4A1 in inflammatory lymphocyte antigen bC (Ly-bC^{high}) and its function in healing after myocardial infarction has also recently been reported [74]. Ly-bC^{high} regulates a biphasic inflammatory and reparative response in the healing process and the loss of NR4A1 impairs healing and macrophages.

Thus, NR4A1 essentially plays a protective role in cardiovascular disease, and the protective effects of NR4A1 and Csn in the high fat/cholesterol mouse model [72] were dissimilar to those observed in db/db and non-genetic models of metabolic disease where NR4A1 promotes metabolic disease [55,56]. It will be important to determine the role of human NR4A1 in these responses prior to clinical applications of NR4A1 ligands.

2.3. NR4A1 and neurological functions

NR4A2 (Nurr1) has been extensively investigated with respect to neuronal function since Nurr1$^{-/-}$ mice exhibit a well characterized selective loss of dopamine biosynthesis in the substantial nigra/ventral tegmental area of the brain but not in hypothalamic neurons [75]. However, there is not only substantial evidence for expression of NR4A1 in various regions of the brain [76,77] but also an increasing number of reports demonstrating the neuronal functions of this receptor [78]. cAMP response element binding protein (CREB) is an important nuclear transcription factor involved in neuroprotection, and results of cell culture and *in vivo* studies indicate that NR4A receptors mediate CREB-dependent neuroprotection [79]. Induced learning in mice by contextual fear conditioning increased expression of NR4A1, NR4A2 and NR4A3 in the hippocampus and similar results were observed for histone deacetylase inhibitor-induced enhanced memory [80]. A recent study delineated differences in the functions of NR4A1 and NR4A2 in the brain; NR4A2 was important for long term memory, object location and recognition, whereas NR4A1 was required only for object location [81]. NR4A1 has also been linked to synaptic remodeling, response to L-DOPA, behavioral changes and dopaminergic loss after administering 1-methly-4-phenyl-1,2,3,6-tetrahychopyridine (MPTP) in mice [82–85]. MPTP-induced loss of dopaminergic neurons is more severe in NR4A1$^{-/-}$ mice compared to wild-type mice, and MPTP-dependent downregulation of NR4A1 is mediated by decreased expression of myocyte enhancer factor 2D (MEF2D) [85]. Patients chronically treated with antipsychotic drugs may develop tardive dyskinesia (TD) and in rodent models of this disease, there is an increase in NR4A1 expression [83,86]. This has also been observed in non-human primates and it has been suggested that NR4A1 may be a target for intervention [86]. Another possible chemotherapeutic role for NR4A1 ligand may for treatment of strokes since NR4A1 is decreased in neural cells deprived of oxygen and glucose, and neural damage is rescued by NR4A1 overexpression [87]. Thus, the development of NR4A1-specific ligands for treatment of some neurological disorders represents both an opportunity and challenge for the future.

2.4. NR4A1 and arthritis

Arthritis is another example of an inflammatory disease, and both NR4A1 and NR4A2 are induced in experimental models of inflammation [88–90]. For example, type II collagen-induced arthritis was significantly decreased in mice overexpressing NR4A1 compared to wild-type mice [88], suggesting another possible therapeutic target for an NR4A1 agonist such as Csn.

2.5. NR4A1 and inflammation and immune responses

The rapid induction of NR4A receptors in response to diverse inflammatory agents and their roles in T-cell receptor-mediated apoptosis has been reviewed [91] and noted in Section 1. Moreover, the roles of NR4A1 in metabolic, cardiovascular and neurological disease and arthritis are associated with inflammatory conditions. With the exception of metabolic disease models, most studies on inflammation and immune responses suggest that although

Please cite this article in press as: S. Safe, et al., Nuclear receptor 4A (NR4A) family – orphans no more, J. Steroid Biochem. Mol. Biol. (2015), http://dx.doi.org/10.1016/j.jsbmb.2015.04.016

NR4A1 is induced under inflammatory conditions, the receptor tends to be protective and is a potential target for NR4A1 agonists. Key recent papers include the observation that (i) NR4A receptors (all 3) play an important role in T-cell development [50], (ii) NR4A1 regulates subsets of genes important for differentiation of Treg cells [92], and (iii) NR4A1 is important for the anti-inflammatory effects of apoptotic cells in macrophages [93].

2.6. NR4A1 and cancer

NR4A1 and its role in cancer have been recently reviewed [38–42] and only the important key concepts will be included in this article. Results of animal studies in which NR4A1 and NR4A3 have been knocked out and subsequent work on cell culture models indicate that NR4A1 is a tumor suppressor for the AML form of leukemia [48,49]. In contrast, studies in other leukemia cell lines suggest a possible oncogenic role for NR4A1 [94] and research on the leukemia-type dependent differences in the function of NR4A1 is ongoing. The expression and function of NR4A1 in solid tumors is consistent in multiple tumor types. For example, NR4A1 is overexpressed in colon, pancreatic, breast (estrogen receptor positive and negative), and lung tumors, and in breast, colon and lung tumor patients high expression of NR4A1 predicts decreased survival [17,95–100]. The functional activity of NR4A1 in cancer has been extensively investigated in cancer cell lines by either knockdown or overexpression, and results have shown that in lung, melanoma, lymphoma, pancreatic, colon, cervical, ovarian, and gastric cancer cell lines, NR4A1 regulates one or more of cancer cell proliferation, survival, cell cycle progression, migration, and invasion [17,96,98–107].

The mechanisms of action of NR4A1 are highly complex and involve both the nuclear and cytosolic receptors. Some of the earliest studies on NR4A1 in cancer cells demonstrated a novel pathway in which the caged retinoid compound CD437 and several analogs and diverse apoptosis-inducing agents induce apoptosis in cancer cell lines by inducing nuclear export of NR4A1 [108–120]. This nuclear export pathway has been linked to formation of a pro-apoptotic mitochondrial NR4A1-bcl2 complex, and this is also observed using peptide mimics and paclitaxel which simulates NR4A1 interactions with bcl2 [121,122]. Cytosolic NR4A1 plays an important role in the mechanism of action of several pro-apoptotic antineoplastic agents including platinum-based drugs [123]. It was also observed that increased expression of chromodomain helicase/adenosine triphosphatase (ATPase) DNA-binding protein 1-like (CHD1L), which inhibits nuclear export of NR4A1, was associated with the increased survival of hepatocellular carcinoma cells [124].

The extranuclear activity of NR4A1 is a drug-induced response which invariably results in the induction of apoptosis; however, results of most knockdown or overexpression studies demonstrate a role for NR4A1 in cell proliferation, survival, migration and invasion. Presumably these responses are primarily due to NR4A1-regulated genes, and results in pancreatic cancer cells have identified genes that fit into each of these categories [101]. Mechanistic studies in colon cancer cells have identified several pathways that are consistent with the pro-oncogenic functions of nuclear NR4A1. Fig. 2 summarizes some of these pathways observed in colon cancer cells. NR4A1 interacts with and inactivates p53 and, based on results of RNAi experiments, this results in activation of mTOR due to decreased expression of p53-regulated sestrin 2 and inactivation of AMPKα [96]. NR4A1 also regulates expression of survivin and other Sp-regulated genes containing GC-rich promoters [17,18], and NR4A1 also regulates redox genes such as isocitrate dehydrogenase1 (IDH1) and thioredoxin domain containing 5 (TXNDC5) to maintain low levels of intracellular stress [18,101]. NR4A1 also activates the pro-invasion gene MMP9 and suppresses E-cadherin [98] and also modulates β-catenin expression through multiple pathways [100,125,126]. These are examples of some NR4A1-regulated genes and pathways in colon cancer cells and other pathways including the cooperative role of NR4A1 in TGFβ-induced epithelial–mesenchymal-transition (EMT) in breast cancer cells [99], demonstrating the pro-oncogenic functions of the receptor. Thus, development of NR4A1 antagonists will be highly advantageous for cancer chemotherapy due to their potential for disabling multiple pro-oncogenic pathways (Fig. 2).

Wu and coworkers identified 3 structurally diverse compounds that bind NR4A1-LBD, namely CsnB and related compounds, TMPA, and 1-(3,4,5-trihydroxyphenyl)nonan-1-one (THPN) (Fig. 1) [34,55,56,127]. Results of modeling and receptor mutation studies

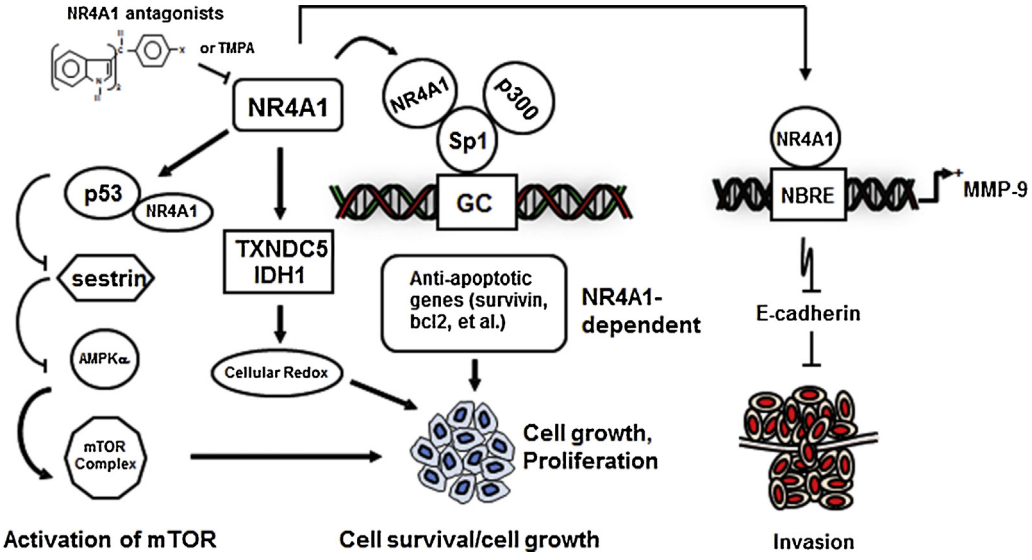

Fig. 2. NR4A1-regulated pathways in cancer cells that are inhibited by C-DIM/NR4A1 antagonists. Treatment of cancer cell lines with C-DIM/NR4A1 antagonists such as DIM-C-*p*PhOH or knockdown of NR4A1 by RNA interference results in inhibition of mTOR signaling by activation of p53, resulting in the induction of sestrin 2 and activation of AMPKα [96]. This is also accompanied by induction of ROS and ER stress through downregulation of TXNDC5 and IDH1 [18,101] and also decreased expression of NR4A1/Sp1-regulated pro-survival/growth promoting genes [17]. Cancer cell invasion is also inhibited by antagonizing NR4A1 which results in decreased expression of MMP9 and E-cadherin [98].

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show that these compounds interact with different amino acid side-chains within the LBD. CsnB exhibits NR4A1 agonist activity in transactivation assays and inhibits cancer cell and tumor growth and these effects are associated with nuclear export of NR4A1 [65]. The antineoplastic activity of THPN is also due to nuclear export of NR4A1 [127]. There is some evidence that TMPA may act as a nuclear NR4A1 antagonist; however, the anticancer activates of this compound has not been characterized [55]. Studies in this laboratory have identified 1,1-bis(3′-indolyl)-1-(*p*-substituted phenyl)methane (C-DIM) compounds that modulate NR4A1-dependent transactivation [17,18,96,97,101,128]. Early studies identified the *p*-methoxy-phenyl analogy (DIM-C-*p*PhOCH$_3$) as a potential NR4A1 agonist in pancreatic cancer cells using rodent derived NR4A1 constructs [128]; however, in subsequent studies using human NR4A1 we observed that this compound was only a weak agonist and most C-DIMs inhibited NR4A1-dependent transactivation [18,39]. In collaboration with the Wu laboratory, we have now shown that many C-DIM analogs including the *p*-hydroxyl, trifluoromethyl, bromo, unsubstituted, cyano, chloro, iodo and carboxymethylphenyl analogs all directly bind the NR4A1-LBD [18]. Modeling studies for the highly active *p*-hydroxyphenyl compound (DIM-C-*p*PhOH) (Kd = 0.11 μM) show a unique interaction with the LBD of NR4A1 that differs from other ligands identified in the Wu laboratory. DIM-C-*p*PhOH and related compounds act directly on nuclear NR4A1 and exhibit NR4A1 antagonist activity, and results in cancer cell lines and tumors show that DIM-C-*p*PhOH is a highly effective anticancer agent [17,18,96,97,101]. As an NR4A1 antagonist, DIM-C-*p*PhOH inhibits the pro-oncogenic NR4A1-dependent pathways outlined in Fig. 2, suggesting that C-DIM compounds and other NR4A1 antagonists represent an important new class of mechanism-based anticancer agents.

3. NR4A2 in cellular homeostasis and disease

The nuclear receptor NR4A2 (Nurr1, HZF-3, RNR1, NOT, DHR38) is the second member of the NR4A family and possesses structural motifs and complex patterns of transcriptional activity similar to NR4A1 and NR4A3. The DNA-binding domain of NR4A2 is over 92% homologous to the same domain of NR4A1 (Nur77), conferring similarities both in sequence identity and function between these receptors [129]. Research over the past two decades has demonstrated activities of NR4A2 associated with energy metabolism, atherosclerosis and vascular function, T-cell receptor (TCR)-mediated apoptosis, inflammatory responses, regulation of the hypothalamic-pituitary axis (HPA) and reproductive processes [59]. Additionally, NR4A2 plays a significant role in development and homeostasis of the central nervous system and has been associated with functional working memory as well as neurological disorders such as Parkinson's disease [78,130]. Like NR4A1 and NR4A3, NR4A2 modulates target gene transcription by binding as a monomer, homodimer or heterodimer with RXR to *cis*-acting response elements such as the NGFI-B-responsive element (NBRE) located in gene promoter regions, which exhibit a canonical AAAGGTCA consensus sequence [131,132]. Additionally, NR4A2-RXR heterodimers bind to a related sequence motif, DR5 (5′-AGGTCANNNAAAGGTCA-3′) in the presence of 9-*cis*-retinoic acid, whereas NR4A2 homodimers bind to another related sequence with the palindromic structure, 5′-TGACCTTTNNNNNAAAGGTCA-3′ [133]. The transcriptional activity of NR4A2 is not limited to transactivation but also includes transcriptional repression or "transrepression" by a mechanism involving recruitment of nuclear co-repressor proteins that stabilize histone-DNA binding and suppress gene expression [134,135]. Genome-wide transcriptomic and ChIP-on-ChIP studies have described a large number of target genes that are regulated by NR4A2 but the effects of NR4A2 activation on gene expression are highly dependent on cell type and on the nature of the signaling event studied. For example, NR4A2 activation can induce apoptosis in cancer cell lines [136,137], but can also stimulate development and maturation of dopaminergic neurons [138–140], as well as block inflammatory responses in macrophage cells [130]. The loss of NR4A2 in mice results in the failure of dopamine neurons to differentiate [77] and like NR4A1, NR4A2$^{-/-}$ mice exhibit many other deficits as outlined below. To understand the regulatory effects of NR4A2, it will therefore be important to elucidate cell-specific signaling mechanisms and to identify distinct protein complexes that associate with either transcriptional activation or repression. Although the effects of NR4A2 are diverse, it has been proposed that NR4A transcription factors are the first-wave transcriptional response to environmental cues that cause diverse adaptive changes in cellular physiology [78].

3.1. NR4A2 and metabolic disease

Because of its effects in regulating genes important for metabolism, small molecular activators of NR4A2 are highly sought after as potential therapeutic agents for metabolic disease. The crystal structure of the human NR4A2 LBD indicates that it does not apparently contain a classical ligand binding cavity, as seen with other NR4A family members and with other steroid hormone receptors, due to the intrusion of side chains from several bulky hydrophobic residues in the region normally occupied by ligands [8]. These structural studies also suggested that the conformation of the NR4A2 LBD might confer a level of constitutive activity, due to the resemblance to the ligand-bound conformation of RXR. Later computer-based modeling of the NR4A2 LBD identified a hydrophobic region opposite the classical co-activator-binding site that is critical for transcriptional activity, as demonstrated by site-directed mutagenesis studies [141]. Mutations in this region reduced or abolished transcriptional activity of the NR4A2 LBD and indicated that proteasome-dependent degradation was important for NR4A2 protein turnover and modulation of the transcriptional effects of the receptor. Although the endogenous ligand for NR4A2 has yet to be identified, a number of compounds have been reported to activate or otherwise modulate the activity of the receptor. Among these, the anti-metabolite cancer drug 6-mercaptopurine, which is widely used for the treatment of acute childhood leukemia and chronic myelocytic leukemia, was shown to induce NR4A2 and NR4A3 through a motif in the N-terminal AF-1 domain of the receptor [142]. However, direct binding of 6-mercaptopurine to NR4A2 remains to be determined. It has also been reported that several benzimidazole compounds have high affinity for the NR4A2, as well as a series of isoxazolopyridinone compounds [30]. Another synthetic small molecule activator of NR4A2, 1,1-bis(3′-indolyl)-1-(*p*-chlorophenyl)methane (C-DIM12), caused ligand-dependent activation of NR4A2 and subsequent poly(ADP-ribose) polymerase (PARP) cleavage and apoptosis in bladder cancer cells that was abolished by RNAi knockdown of NR4A2 [136]. Recent modeling and receptor binding studies with this class of compounds have identified several C-DIM analogs that directly binding to NR4A1 in a groove along the co-activator interface of the LBD [18]. This domain is highly conserved between NR4A family members, suggesting that selected C-DIM compounds with small, polar substituents on the phenyl ring could modulate NR4A2 transcriptional activity through direct binding to the receptor

The ability of NR4A2 to regulate the expression of multiple genes associated with metabolism and gluconeogenesis suggests that this factor may also be an important regulator of metabolic disease. The hepatic expression of NR4A2 is induced by cAMP in

response to glucagon as well as a number of other compounds acting on the β-adrenoreceptor signaling axis, including fatty acids, glucose, insulin, cholesterol and thiazolidinediones [30]. NR4A2, as well as NR4A1 and NR4A3, are induced in rat liver after dietary restriction, underscoring the importance of the NR4A receptor subgroup with dietary inputs positively regulating metabolism [143]. Furthermore, dietary restriction in these studies also increased expression of Ucp-3, Ampkg3, Pgc-1a and Pgc-1b in muscle, which is consistent with increased activity of both NR4A1 and NR4A3 and which positively correlated with improved glucose utilization and insulin sensitivity. Other metabolically associated genes regulated by NR4A2 include the ATP-binding cassette subfamily G members 5 and 8 (AbcG5/8), Apolipoproteins B and E (ApoB/E), fatty acid synthase (Fas), fructose-1,6-bisphosphatase 1 and 2 (Fbp1/2), glucose transporter 4 (Glut4), uncoupling protein 2 and 3 (Ucp2/3), and the peroxisome proliferator-activated receptor-γ (Pgc1a), as extensively reviewed by Pearen and Muscat [30]. The NR4A subgroup, including NR4A2, is likely critically important for glucose utilization and for maintaining homeostasis in cholesterol and fatty acid metabolism. Small molecular ligands of NR4A2 could therefore be effective in treating aspects of metabolic disease and are being intensively studied for this purpose.

3.2. NR4A2 and cardiovascular disease

Atherosclerosis is characterized by hardening of arteries as a result of formation of plaques that compromises normal blood flow over time. Atherosclerotic plaques contain fat, cholesterol and calcium deposits that stimulate a proliferative response in smooth muscle cells within the media of arteries, resulting in further constrictions to blood flow that may lead to myocardial infarction, stroke and death. Activation of endothelial cells at atherosclerotic plaques attracts circulating monocytes that represent an early event in the development of atherosclerotic lesions. NR4A receptors are moderately induced in atherosclerotic endothelial cells and macrophages and it has been proposed that amongst this receptor subfamily, NR4A3 promotes the development of atherosclerotic lesions, whereas NR4A1 and NR4A2 attenuate atherosclerosis [132]. NR4A2 also appears to have an anti-mitogenic effect in smooth muscle cells, which antagonizes the formation of atherosclerotic plaques [144]. The ability of NR4A2 to inhibit NFκB-dependent expression of inflammatory genes in macrophages may also contribute to the anti-atherogenic activity of this factor [59,135]. This activity may be of particular importance, given that activated macrophages release cytokines and growth factors that aggravate local inflammation and activate underlying smooth muscle cells, leading to excessive uptake of lipids and the transition of macrophages into lipid-laden foam cells that remain resident in the atherosclerotic lesion [58]. Supporting the role for NR4A2 in protection against atherogenesis, it was discovered that NR4A2 is negatively regulated by miR-145 in smooth muscle cells and that mice lacking miR-145 are resistant to the development of atherosclerotic plaques, owing to their high expression of NR4A2 [145]. Additionally, studies in human macrophages using lentiviral-mediated overexpression or knockdown of NR4A2 demonstrated that NR4A2 inhibited the uptake of oxidized LDL by macrophages and reduced the expression of pro-inflammatory cytokines and chemokines [146]. Collectively, these data support a role for NR4A2 in protection against cardiovascular disease.

3.3. NR4A2 and neurological function

NR4A2 has pleiotropic effects on gene expression in the brain which are highly dependent on cell type and on the specific extracellular signal or stressor encountered. Studies in human neural SK-N-AS cells were conducted in which a number of stable clonal lines were constructed with graded NR4A2 gene expression to approximate levels of NR4A2 seen in dopaminergic neurons present in human substantia nigra [147]. Transcriptomic data acquired from these NR4A2-expressing clonal lines revealed that the effects of NR4A2 on target genes varied considerably as a function of its concentration. Nearly one-fifth of NR4A2-responsive transcripts showed bidirectional changes with increasing NR4A2 expression where some genes were induced and others suppressed. Transcripts that were induced by increasing concentrations of NR4A2 included genes such as the neurodevelopment factors Crmp1, Kif1a and Tubb2a, whereas a number of transcripts were decreased at all higher concentrations of NR4A2, including the NFκB-related transcripts (Nfkb1, Nfkb1a), TNF-related transcripts (Tnf Tnfip1, Tnf4sf4), and the peroxisome proliferator-activated receptor γ (Pgc1) [147]. These data strongly suggest that NR4A2 exerts concentration-dependent effects that dramatically influence transcriptional programs in neural cells.

NR4A2 is widely expressed throughout the brain and is present in telencephalic structures such as the cortex and hippocampus, although it is most well studied in context of its effects in dopaminergic neurons. As an immediate-early gene encoding a member of the steroid-thyroid hormone receptor family, NR4A2 is also rapidly induced following stress and injury in the CNS. In postnatal mice exposed to the glutmate receptor agonist, kainic acid, NR4A2 protein levels were rapidly induced in pyramidal neurons in the CA1 and CA3 layers of the hippocampus, as well as more transiently in the dentate gyrus, a region generally more resistant to neuronal in injury from kainic acid exposure [148]. NR4A2 is also involved with memory and learning, which may be mediated in part by MAP kinase signaling pathways that alter its phosphorylation state and its nuclear localization, described in studies where stimulation of ionotropic glutmate receptors (AMPA, NMDA) resulted in increased phosphorylation of NR4A2 and its export from the nucleus [78]. Interactions between NR4A2 and the cyclic-AMP response element binding protein (CREB) are important for memory and knockdown of NR4A in the hippocampus using antisense oligonucleotides impaired long-term memory and reversal learning in an appetitive spatial learning task [149].

In dopaminergic brain regions, particularly the substantia nigra (SN) and the ventral tegmental area (VTA), NR4A2 is important both for development and homestasis of dopamine producing neurons. This was initially demonstrated by studies using NR4A2 knockout mice, in which mice lacking NR4A2 failed to generate midbrain dopaminergic neurons, were hypoactive and died during the early postnatal period [77]. NR4A2 is also required for maintenance and maturation of adult midbrain dopamine neurons [139], in part, through its regulation of dopamine synthesis and metabolism [150,151]. Mice heterozygous for NR4A2 display an age-dependent decline in the number of dopaminergic neurons in the SN compared to wild-type mice and also exhibit a decrease in peak evoked DA release that is only partly compensated by increased expression of the dopamine transporter [152]. The age-related decline in neurological function in heterozygous NR4A2 knockout mice correlates with the effects of decreased NR4A2 expression in models of Parkinson's disease (PD), where reduced expression of NR4A2 increases the vulnerability of mesencephalic dopamine neurons to injury from 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [153]. In a study of 201 individuals affected with PD and 221 age-matched unaffected controls, two mutations were identified that associated with PD and mapped to the first exon of NR4A2 [154]. A subsequent study in 278 patients with PD, 166 healthy controls (HC), and 256 neurological disease controls revealed that lower expression of NR4A2 resulted in a significant increase in the risk for developing

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PD [155]. Selected point mutations in human NR4A2 are also implicated in PD by decreasing expression of tyrosine hydroxylase, the rate limiting enzyme in dopamine synthesis [156]. NR4A2 also regulates the expression of α-synuclein, the major protein constitutent of Lewy body aggregates in PD, and decreased expression of NR4A2 transcriptionally increases α-synuclein expression [157]. The importance of NR4A2 in PD was also highlighted in recent studies using a synthetic small molecular activator of NR4A2, 1,1-bis(3′-indolyl)-1-(*p*-chlorophenyl)methane, which protected against MPTP-induced loss of dopaminergic neurons in a mouse model of PD and increased expression and nuclear localization of NR4A2 in the SN [158]. Collectively, these data indicate that NR4A2 is an important factor regulating multiple physiologic functions in the CNS but also suggest that this transcription factor in important in protection against oxidative and inflammatory stress relevant to neurodegenerative disorders including PD.

3.4. NR4A2 in inflammatory and immune responses

NR4A2 appears to have both constitutive and inducible anti-inflammatory activity in monocyte/macrophage lineage immune cells, as well as in brain glial cells including both astrocytes and microglia. This anti-inflammatory activity appears to be directed towards the NFκB signaling pathway in response to inflammatory stimuli such as tumor necrosis factor α (TNFα) and bacterial lipopolysaccharide (LPS). Following exposure to TNFα or LPS, the p65 (RelA) subunit of NFκB rapidly translocates to the nucleus, where its histone deacetylase activity and subsequent phosphorylation by GSK3β facilitates opening of chromatin and removal of constitutively bound nuclear co-repressor complexes [135]. These studies also report that sumoylation of NR4A2 on K577 of the LBD is essential for this transrepressive activity. Earlier studies support this conclusion, because K577 in the NR4A2 LBD is part of a consensus SUMO-modification sequence and mutation of this lysine to arginine results in decreased transcriptional activity, suggesting that sumoylation of K577 is important for transcriptional modulation by NR4A2 [141]. In brain glial cells, the anti-inflammatory effects of NR4A2 are mediated by docking to NFκB-*p*65 on target inflammatory gene promoters, followed by recruitment of the CoREST co-repressor complex, resulting in clearance of NFκB-*p*65 and transcriptional repression [135]. Because inflammatory activation of glial cells is critical to the progressive loss of dopaminergic neurons in PD, these studies suggest that NR4A2 protects against neuronal loss in part by limiting the production of neurotoxic mediators by microglia and astrocytes. NR4A2 also plays a pro-inflammatory role in synoviocytes associated with arthritis and a recent report that NR4A2 regulation of prolactin expression contributes to this response [159].

NR4A2 also influences maturation and differentiation of Th17 T-cells and may thereby have a role in both autoimmunity and in resolution of infections [160]. NR4A2, but not NR4A1 or NR4A3, is upregulated in rheumatoid arthritis, where its expression is induced by PGE2, IL-1β, and TNF-α [161], suggesting that this factor may have broad effects in limiting inflammatory responses through its function as a transrepressor of the NFκB pathway. Additionally, NR4A2 regulates expression of the Forkhead transcription factor Foxp3, which is important for differentiation of regulatory T cells (Treg cells) and is mediated through direct interaction of NR4A2 with Runx1 [30]. Induction of NR4A2, along with other soluble and cell-surface mediators with anti-inflammatory activities (*e.g.*, IL-10, TGF-β, resolvins, ligands for TAM receptors), is important to attenuate responses to inducers or amplifiers of inflammation [130]. Based on these and other studies, NR4A2 appears to be broadly important for regulating both inflammation and resolution of inflammatory signaling in activated immune cells and glial cells.

3.5. NR4A2 and cancer

A number of receptor knockdown or overexpression studies both *in vivo* and in cancer cell lines demonstrate that NR4A orphan receptors exhibit pro-oncogenic or tumor suppressor-like activity that is dependent on the type of tumor [38]. NR4A receptors have been shown to enhance cell proliferation, apoptosis, and differentiation in a tissue-specific context [29]. NR4A2 is upregulated in normal breast epithelium compared to breast cancer cells, suggesting an inverse correlation between breast cancer and the level of NR4A2 expression [162]. These studies also reported that short hairpin RNA (shRNA)-mediated silencing of NR4A2 gene expression in breast tumor xenografts in mice significantly reduced tumor growth [132]. Immunohistochemical analyses of human prostate cancer biopsies indicated that expression of NR4A2 was significantly higher than in normal controls, suggesting an inverse relationship between expression of NR4A2 and tumor growth [163]. Likewise, silencing of NR4A2 expression *in vitro* in prostate cancer cells reduced cell proliferation, invasion and migration, indicating that NR4A2 could a biomarker for the progression of breast and prostate cancer [132].

NR4A2 is more highly expressed in estrogen receptor-positive breast and bladder tumors compared with normal tissue, and higher levels of cytoplasmic NR4A2 were a prognostic factor for high tumor grade, decreased survival, and increased distant metastasis in a cohort of bladder cancer patients [38]. NR4A2 is also more highly expressed in prostate tumors compared to normal prostate and correlates with tumor classification and Gleason score as a negative prognostic factor [163]. Small molecules that stimulate the inhibitory effects of NR4A2 may have promise in treating cancer, demonstrated by the effects of the NR4A2-acting compound 1,1-bis(3′-indolyl)-1-(*p*-chlorophenyl)methane (C-DIM12 or DIM-C-*p*PhCl) which induced TRAIL protein expression and PARP cleavage in bladder cancer cells that was significantly decreased by inhibition of NR4A2 with RNAi [136]. Interestingly, overexpression of NR4A2 in colorectal cancer cells revealed that ectopic expression of NR4A2 increased resistance to the chemotherapeutic agents 5-fluorouracil and oxaliplatin and attenuated the chemotherapeutic-induced apoptosis [164]. Using tissue microarray analysis, these studies also found that NR4A2 expression was increased in colorectal cancer specimens collected from 51 adenomatous colorectal cancers, 14 familial adenomatous polyposis colorectal cancers, 17 stage IV colorectal cancers with adjacent mucosa, and 682 stage I–III colorectal cancers. Increased expression of NR4A2 was related to protein kinase A activation and was correlated with chemoresistance [164]. These data demonstrate that NR4A2 expression predicts poor survival and drug resistance in various cancers, including breast, prostate, bladder and colon cancers.

4. NR4A3 in cellular homeostasis and disease

The nuclear receptor NR4A3 (Nor1, TEX, MINOR, CHN) is the third member of the NR4A family and shares many of the same characteristics reported for NR4A1 and NR4A2. NR4A3-mediated transactivation and interactions with various *cis*-elements, except that unlike NR4A1 and NR4A2, NR4A3 does not form a heterodimer with RXR [15,16]. Although there is some redundancy in the functions of the three NR4A receptors since these receptors are induced as early immediate genes by some of the same stressors [29,30], each receptor also exhibits unique functions. One study reported that loss of NR4A3 in mice was embryo-lethal [165]; however, subsequent studies indicate that NR4A3$^{-/-}$ mice survive

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but exhibit deficits in the semicircular canals of the inner ear and in hippocampal development [166,167]. This latter response can lead to several neuronal deficits and enhanced kainic acid-induced seizures. As indicated previously, double knockout NR4A1$^{-/-}$/NR4A3$^{-/-}$ mice rapidly develop acute AML-type leukemia and have been designated as tumor suppressors for this type of cancer [48,49]. Moreover, studies on knockdown of NR4A3 and other NR4A receptors demonstrate a role for NR4As in immune homeostasis and regulation of T-cell development and aspects of metabolic disease [50,52]. Muscat and coworkers have previously reviewed the physiological and pathophysiological roles of NR4A3 and other NR4A receptors [29,30], and this article will highlight some of these functions and more recent studies.

4.1. NR4A3 and metabolic disease

Knockdown of NR4A3 in c2c12 skeletal muscle cells resulted in changes in gene expression consistent with a shift from oxidative to anaerobic gene expression [168], and subsequent studies in NR4A3-overexpressing mice demonstrated that NR4A3 increases type II muscle fibers and resistance to fatigue [169]. A role for NR4A3 in high *vs*. low running capacity in rodents was also reported [170]. NR4A3 induced cAMP in hepatocytes and in mouse liver in fasted mice [53] and levels were upregulated in obese patients [52]. The role of this receptor in mouse models of obesity and T2DM have not been extensively investigated.

4.2. NR4A3 and cardiovascular disease

NR4A3 is expressed in atherosclerotic lesions and is induced by diverse stressors in smooth muscle cells [69,171–174], and knockdown experiments in smooth muscle cells suggest that this receptor plays a role in proliferation of these cells [174,175]. NR4A3-induced proliferation has been linked to regulation of cyclins D1 and D2 [174,175] and S-phase kinase-associated protein 2 (Skp2) which results in decreased expression of the cyclin-dependent kinase inhibitor p27 [176]. A recent study showed that miR-638 is also a key regulator of smooth muscle cell proliferation by targeting NR4A3 which results in silencing of NR4A3-regulated genes involved in cell proliferation [177]. These results coupled with data from a mouse model overexpressing NR4A3 in smooth muscle cells demonstrate that in contrast to NR4A1, NR4A3 serves to enhance neointintima hyperplasia. A recent study showed that NR4A3 inhibited NFκB signaling in vascular smooth muscle cells demonstrating an anti-inflammatory function in this cell type [178]. NR4A receptors including NR4A3 are induced by VEGF in endothelial cells [69] and this proliferative response is inhibited after NR4A3 knockdown [171,179]. A recent study reported that NR4A3 regulated vascular cell adhesion molecule-1 (VCAM-1) in endothelial cells through direct binding to an NBRE promoter element [180]. NR4A3 plays a role in monocyte adhesion and in an *in vivo* model for atherosclerosis using ApoE$^{-/-}$/NR4A3$^{-/-}$ mice, it was confirmed that NR4A3 regulates recruitment of monocytes to the vascular wall. NR4A3 facilitates macrophage recruitment, whereas the opposite response is observed for NR4A1 [72].

4.3. NR4A3 and neurological functions

NR4A genes have important neuronal functions [80–82] and studies with NR4A3$^{-/-}$ mice show specific hippocampal functions for this receptor. NR4A3 is a CREB-regulated gene and plays a role in memory enhancement by HDAC inhibitors [80]. More recent studies show differential expression of NR4A receptors [181,182] and studies on dopamine neurons showed that in the ventral tegmental area, haloperidol rapidly induced NR4A3 and NR4A1 (but not NR4A2) and this was accompanied by induction of tyrosine hydroxylase and the dopamine transporter-mRNA. Functional studies also showed that NR4A3 expression in the Wistar–Kyoto rat contributed to depressive behavior [183]. Moreover, polymorphisms within the NR4A3 gene are correlated with nicotine addiction in patients with mental health disease [184], and it is possible that NR4A polymorphisms may also be associated with other receptor mediated health problems.

4.4. Inflammation and immune responses

NR4A3 like all NR4A receptors is induced by stressors and is upregulated by inflammatory conditions and also plays an integral role in T-cell receptor-induced apoptosis [29,30,49,50]. Knockdown of NR4A1, NR4A2 and NR4A3 (combined) in mice resulted in death within 3 weeks and among the double knockout mice, only the NR4A1$^{-/-}$/NR4A3$^{-/-}$ mice died within 3–4 weeks [50]. Moreover, in this same study development of Treg cells was also decreased and it was concluded that "NR4A1 and NR4A3 were the main contributors to Treg cell homeostasis and the prevention of autoimmunity" [50].

4.5. NR4A3 in cancer

The combined loss of NR4A3 and NR4A1 in mice results in acute AML-type leukemia [48,49] and HDAC-inhibitor mediated apoptosis in AML cells is accompanied by induction of NR4A3 [185]. These results demonstrate a role for NR4A3 (in combination with NR4A1) as a tumor suppressor for AML; however, the function of this receptor in other leukemias has not been determined. NR4A3 is downregulated in nasopharyngeal carcinomas due to promoter hypermethylation, and in cell lines overexpression of NR4A3 decreased cell proliferation and colony formation and this was consistent with tumor suppressor activity [186,187]. NR4A3 is one of only a few NRs overexpressed in ER-positive and ER-negative breast tumors, and a recent report shows that NR4A3 expression is higher in triple negative *vs*. luminal tumors [95,188]; however, the function of this receptor in breast cancer has not yet been determined. NR4A3 is also overexpressed in human hepatocellular carcinomas and induces hepatocyte proliferation, and the function of this gene in liver cancer cells has not been determined [189]. Genomic studies in horses have also linked NR4A3 to susceptibility to melanoma [190] but expression and functions in human melanomas have not been determined. Prostaglandin A2 is the only reported NR4A3 ligand [191], and future clinical applications for targeting NR4A3 will require additional insights on tumor-specific functions of this gene and development of new ligands.

5. Summary

NR4A1, NR4A2 and NR4A3 are orphan nuclear receptors and immediate early genes induced by multiple stressors. All three receptors bind the same genomic *cis*-elements; however, their distinct differences in activities are due, in part, to their more unique N- and C-terminal domains that differentially interact with various cofactors and ligands and their tissue-specific expression. Complete and tissue-specific knockout mouse models uniquely distinguish between the different roles for these receptors in metabolic, cardiovascular, neurological, immune and inflammatory functions, cancer and related diseases. Although endogenous ligands for NR4A receptors have not been identified, several recent studies have identified structurally diverse compounds that bind and activate or inactivate nuclear NR4A1 or induce nuclear export of NR4A1, and these compounds show some promise in the treatment of conditions such as metabolic diseases and cancer. For example, a recently published paper showed that the pro-fibrotic

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effects of transforming growth factor-β (TGF-β) signaling was inhibited by NR4A1 which recruits inhibitory chromatin-modifying complexes to the promoters of TGF-β-regulated genes [192]. The NR4A1 agonist cytosporone B inhibits experimentally-induced fibrosis in multiple tissues "demonstrating the first proof of concept for targeting NR4A1 in fibrotic diseases" [192]. Future clinical applications of NR4A ligands will require the synthesis and development of ligands specific for NR4A1, NR4A2 and NR4A3 and characterization of selective NR4A modulators that can be used for their tissue-specific agonist/antagonist activities.

Disclosure

There are no conflicts of interest to disclose.

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Please cite this article in press as: S. Safe, et al., Nuclear receptor 4A (NR4A) family – orphans no more, J. Steroid Biochem. Mol. Biol. (2015), http://dx.doi.org/10.1016/j.jsbmb.2015.04.016

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Please cite this article in press as: S. Safe, et al., Nuclear receptor 4A (NR4A) family – orphans no more, J. Steroid Biochem. Mol. Biol. (2015), http://dx.doi.org/10.1016/j.jsbmb.2015.04.016

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Nuclear Receptor 4A1 (NR4A1) as a Drug Target for Breast Cancer Chemotherapy

Journal:	*Endocrine-Related Cancer*
Manuscript ID:	Draft
Manuscript Type:	Research Paper
Date Submitted by the Author:	n/a
Complete List of Authors:	Hedrick, Eric; Texas A&M University, Veterinary Physiology & Pharmacology Lee, Syng-Ook; Keimyung University, Food Science & Technology Doddapaneni, Ravi; Florida State University, Pharmaceutics Singh, Mandip; Florida A&M University, Pharmaceutics Safe, Stephen; Texas A&M University, Veterinary Physiology & Pharmacology
Keywords:	Breast, NR4A1, indole derivative, antagonists

Nuclear Receptor 4A1 (NR4A1) as a Drug Target for Breast Cancer Chemotherapy

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Running Title: Breast cancer inhibition by NR4A1 antagonists

Keywords: NR4A1, indole derivative, antagonists

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ABSTRACT

The orphan nuclear receptor 4A1 (NR4A1) is overexpressed in mammary tumors and breast cancer cell lines, and the functional activity of this receptor was investigated by RNA interference with oligonucleotides targeted to NR4A1 (siNR4A1) and by treatment with NR4A1 antagonists. Breast cancer cells were treated with NR4A1 antagonists or transfected with siNR4A, and effects on cell proliferation and apoptosis and specific genes associated with these responses were investigated in MCF-7, SKBR3 and MDA-MB-231 cells and in athymic nude mice bearing MDA-MB-231 cells as xenografts. Transfection of MCF-7, MDA-MB-231 and SKBR3 breast cancer cells with siNR4A1 decreased cell proliferation and induced apoptosis in these cell lines. Transfection of breast cancer cells with siNR4A1 also decreased expression of Sp-regulated genes including *survivin*, *bcl-2* and epidermal growth factor receptor, inhibited mTOR signaling in MCF-7 cells that express wild-type p53, and activated oxidative and endoplasmic reticulum stress through downregulation of thioredoxin domain-containing 5 (TXNDC5) and isocitrate dehydrogenase 1 (IDH1). 1,1-Bis(3'-indolyl)-1-(*p*-substituted phenyl)methanes (C-DIMs) are NR4A1 ligands that act as NR4A1 antagonists, and treatment with selected analogs also inhibited breast cancer cell and tumor growth and induced apoptosis, and the effects of C-DIM/NR4A1 antagonists were comparable to that observed after NR4A1 knockdown. Results with siNR4A1 or C-DIMs/NR4A1 antagonists in breast cancer cells and tumors were similar to those previously reported in pancreatic, lung and colon cancer cells and demonstrate the potential clinical applications of NR4A1 antagonists in patients with tumors that overexpress this receptor.

INTRODUCTION

Nuclear receptor 4A1 (NR4A1, Nur77, TR3) is a member of the NR4A orphan receptor sub-family of nuclear receptors and the NR4A receptors (NR4A1, NR4A2 and NR4A3) play essential roles in metabolic processes, inflammation, vascular function, steroidogenesis and the central nervous system (Lee, et al. 2011; Maxwell and Muscat 2006; Pearen and Muscat 2010). NR4A1 is overexpressed in multiple tumors and cancer cell lines and results of receptor knockdown by RNA interference (RNAi) demonstrate that in solid tumors the receptor is pro-oncogenic and regulates cell growth and survival (Bras, et al. 2000; Kolluri, et al. 2003; Lee, et al. 2010; Lee, et al. 2012; Lee, et al. 2014a; Lee et al. 2011; Uemura and Chang 1998; Wu, et al. 2011; Zeng, et al. 2006). Several pro-apoptotic agents including phorbol esters and adamantyl-derived retinoids induce expression and nuclear export of NR4A1 which subsequently binds mitochondrial bcl-2 to form a pro-apoptotic complex that decreases mitochondrial membrane potential (Li, et al. 2000; Lin, et al. 2004; Zhang 2007). This has led to development of peptide mimics that convert bcl-2 into an apoptotic complex and similar results have reported for the taxane-derived anticancer agent paclitaxel (Ferlini, et al. 2009; Kolluri, et al. 2008). Cytosporone B and related analogs have been identified as ligands for NR4A1 (Liu, et al. 2010; Zhan, et al. 2008), and two additional compounds [ethyl 2-[2,3,4-trimethoxy-6-(1-oct-anoyl)phenyl]acetate and 1-(3,4,5-trihydroxyphenyl)no-nan-1-one] also bind NR4A1(Wang, et al. 2014; Zhan, et al. 2012). Ethyl 2-[2,3,4-trimethoxy-6-(1-oct-anoyl)phenyl]acetate inactivates nuclear NR4A1, whereas 1-(3,4,5-trihydroxyphenyl)no-nan-1-one and cytosporone B induce nuclear export of NR4A1.

Studies in this laboratory have been investigating a series of 1,1-bis(3-indolyl)-1-(*p*-substituted phenyl)methane (C-DIM) analogs and their effects on NR4A1 and NR4A1-dependent transactivation (Chintharlapalli, et al. 2005; Cho, et al. 2010; Lee et al. 2010; Lee et al. 2012; Lee, et al. 2009; Lee et al. 2014a; Lee, et al. 2014b). Since NR4A1 exhibits pro-oncogenic activity, we have been focused on identification of C-DIMs that inactivate NR4A1 and the *p*-hydroxyphenyl analog (DIM-C-pPhOH) was characterized as a compound that inactivated nuclear NR4A1 in cancer cell lines and this was not accompanied by nuclear export of NR4A1 (Lee et al. 2010; Lee et al. 2012; Lee et al. 2014a). Subsequent studies comparing the effects of DIM-C-pPhOH and knockdown of NR4A1 (siNR4A1) by RNA interference identified three major pro-oncogenic pathways and associated genes regulated by NR4A1 that were inhibited by DIM-C-pPhOH; 1) NR4A1 regulates expression of genes such as survivin through interactions with specificity protein 1 (Sp1) bound to their proximal GC-rich promoters (Lee et al. 2010); 2) NR4A1 inactivates p53 to enhance mTOR signaling in lung and colon cancer cells expressing wild-type p53 (Lee et al. 2012; Lee et al. 2014b); and 3) NR4A1 regulates expression of thioredoxin domain-containing 5 (TXNDC5) and isocitrate dehydrogenase 1 (IDH1) to maintain low levels of oxidative stress (Lee et al. 2014a) (Fig. 1A).

Recent studies show that NR4A1 is overexpressed in ER-positive and ER-negative breast tumors (Muscat, et al. 2013), and NR4A1 expression in breast tumors is correlated with decreased relapse-free survival (Zhou, et al. 2014). Results of NR4A1 overexpression in breast cancer cells suggest that NR4A1 may be anti-migratory (Alexopoulou, et al. 2010); however, a recent study reported pro-migratory activity for

this receptor (Zhou et al. 2014). Research in this laboratory has demonstrated pro-oncogenic functions of NR4A1 in pancreatic, colon and lung cancer cells, and this study investigates the functions of this receptor in breast cancer cells and the effects of C-DIM/NR4A1 antagonists. The results clearly demonstrate the pro-oncogenic functions of NR4A1 in breast cancer and demonstrate that C-DIM/NR4A1 antagonists represent a potential novel approach for treating breast cancer patients that overexpress this orphan receptor.

MATERIALS AND METHODS

Cell lines and antibodies. MCF-7, MDA-MB-231, and SKBR3 human breast cancer cell lines were purchased from American Type Culture Collection (Manassas, VA) and were kept frozen until initiation of these studies. The cells were received at low passage (< 15) and new frozen stocks were used every 6-8 weeks. The three cell lines were authenticated by Biosynthesis (Lewisville, TX) on February 3, 2015. Cells were maintained 37°C in the presence of 5% CO_2 in Dulbecco's modified Eagle's medium/Ham's F-12 medium with 10% fetal bovine serum with antibiotic. β-Actin antibody and Dulbecco's Modified Eagle's Medium were purchased from Sigma-Aldrich (St. Louis, MO). Sp1 antibody was purchased from Millipore (Temecula, CA); caspases 7 and 8, sestrin 2, bcl2, CHOP, ATF4, IDH1, and epidermal growth factor receptor (EGFR) antibodies were purchased from Santa Cruz Biotech (Santa Cruz, CA). Caspase 3, cleaved poly ADP ribose polymerase (c-PARP; 9541), phospho mTOR, mTOR, phospho AMPKα, AMPKα, phospho p70S6K, p70S6K, phospho S6RP, S6RP, phospho 4EBP1, 4EBP1, and survivin antibodies were purchased from Cell Signaling Technologies (Danvers, MA). TXNDC5 antibody was purchased from Genetex (Irvine, CA). XBP-1s and phospho PERK were obtained from Biolegend (San Diego, CA). Apoptotic, Necrotic, and Healthy Cells Quantification Kit was purchased from Biotium (Hayward, CA). Cells were visualized under an EVOS fl, Fluorescence microscope, from Advanced Microscopy Group using a multiband filter set for FITC, rhodamine, and DAPI. The C-DIM compounds were prepared as previously described (Lee et al. 2010; Lee et al. 2012; Lee et al. 2014a).

Cell proliferation assay. MCF-7, MDA-MB-231, and SKBR3 breast cancer cells (1.0×10^5 per well) were plated in 12 well plates and allowed to attach for 24 hr and cells were treated with 1,1-bis(3'-indolyl)-1-(*p*-carboxymethylphenyl)methane (DIM-C-pPhCO_2Me) in dimethyl sulfoxide (DMSO) for 24 or 48 hr or transfected with siNR4A1 or iGL2 (control siRNA) in lipofectamine for 72 hr. Cells were then trypsinized and counted using a Coulter Z1 cell counter and growth inhibition was determined. Each experiment was carried out in triplicate, and results were expressed as the mean ± SE for each set of experiments. Cells were also treated with C-DIMs after NR4A1 knockdown.

Annexin V staining. MCF-7, MDA-MB-231, and SKBR3 cells(1.0×10^5 per well) were seeded in 2-well Nunc Lab-Tek chambered B#1.0 Borosilicate coverglass slides from Thermo Scientific and were allowed to attach for 24 hr. The medium was then changed to DMEM/Ham F-12 medium contained 2.5% charcoal-stripped fetal bovine serum, and either DMSO or DIM-C-pPhCO_2Me (15 μM) was added for 24 hr. For siRNA treatment, cells were transfected with iGL2 or 100 nm siNR4A1 (1 or 2) for 72 hr. Apoptosis was analyzed by apoptotic and necrotic assay kit (Biotium CA), which contained fluorescein isothiocyanate-annexin-V, ethidium homodimer III and Hoechst 3342. Apoptosis, necrotic and healthy cell detection kit was used according to the manufacturer's protocol and cells were visualized under an EVOS fl, fluorescence microscope, from Advanced Microscopy. The proportion of apoptotic cells was determined by the amount of green fluorescence observed in the treatment groups relative and normalized to control group.

Western blot analysis. Breast cancer cells (3.0 x 10^5 per well) were seeded in Dulbecco's modified Eagle's medium/Ham's F-12 medium in six well plates. Cells were allowed to attach for 24 hr and treated with varying concentrations of DIM-C-pPhCO_2Me for 24 hr or with 100 nm of siNR4A1 for 72 hr. Cells were lysed with high salt lysis buffer (with protease inhibitor cocktail) and quantitated with Bradford reagent. Lysates were then analyzed by SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane by wet electroblotting. Membranes were then incubated with primary and then followed by secondary antibody. Western blot analysis was determined as described and Immobilon western chemiluminescence substrates (Millipore, Billerica, MA) were used to develop images captured on a Kodak 4000 MM Pro image station (Molecular Bioimaging, Bend, OR).

Small interfering RNA interference assay. Breast cancer cells were seeded (1.2 x 10^5 per well) in six well plates in Dulbecco's modified Eagle's medium/Ham's F-12 medium supplemented with 2.5% charcoal-stripped fetal bovine serum and left to attach for 24 hours. Knockdown of NR4A1 was carried out using Lipofectamine 2000 reagent according to the manufacture's protocol. Small inhibitory RNAs and GL2 (non-specific oligonucleotide) were prepared and purchased Sigma-Aldrich (St. Louis MO). The siRNA complexes used in the study are as follows: siGL2-5', CGU ACG CGG AAU ACU UCG A; siNR4A1 (1)-SASI_Hs02_00333289; siNR4A1 (2)-SASI_Hs01_00182072

Generation and measurement of ROS. Cellular ROS levels were ascertained using the cell permeable probe CM-H_2DCFDA (5-(and-6)-chloromethyl-2'7'-dichlorodihydrofluorescein diacetate acetyl ester) from Invitrogen (Grand Island, NY). Following treatment of the cells for 12 or 24 hr with DIM-C-pPHCO_2Me or siNR4A1 for

72 hr, cells were plated on a 6-well culture plate were trypsinized, neutralized, then loaded with 10 μM of probe for 20 min, washed once with serum free medium, and then ROS was measured by flow cytometry using Accuri's C6 Flow Cytometer (Accuri, Ann Arbor, MI).

TNBC Orthotopic Xenograft model. Female BALB/c nude mice (6-8 weeks old) were obtained (Charles River Laboratory, Wilmington, MA) and maintained under specific pathogen-free conditions, housed in isolated vented cages and allowed to acclimate for one week with standard chow diet. The animals were housed at Florida A&M University in accordance with the standards of the Guide for the Care and Use of Laboratory Animals and the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). The protocol of the animal study was approved by the Institutional Animal Care and Use Committee (IACUC), Florida A&M University, Fl. MDA-MB-231 cells ($1X10^6$ cells) were detached, resuspended in 100 μl of phosphate-buffered saline with matrigel (BD Bioscience, Bedford, MA), and implanted subcutaneously in the mammary fat pad of mice. When tumors reached about 40-50 mm^3 size, the animals were randomized into control and treatment groups (6 animals per group) and mice were treated with placebo or DIM-C-pPhCO_2Me or 1,1-bis(3'-indolyl)-1-(*p*-cyanophenyl)methane (DIM-C-pPhCN) (50 mg/kg/d) in nano liquid carrier (administered in sodium carboxymethyl cellulose) by oral gavage every second day for 4 weeks. Tumor volumes and weights, and body weight were determined; the tumor size was measured using Vernier calipers, and the tumor volume was estimated by the formula: tumor volume (mm^3) = (L X W^2) x ½, where L is the length and W is the width

of the tumor. Tumor lysates were obtained and analyzed for protein expression by western blots.

Statistical analysis. Statistical significance of differences between the treatment groups was determined by Student's *t* test. The results are expressed as means with error bars representing 95% confidence intervals for 3 experiments for each group unless otherwise indicated, and a *P* value less than 0.05 was considered statistically significant. All Statistical tests were 2-sided.

RESULTS

1. Inhibition of cell proliferation by siNR4A1 and DIM-C-pPhCO$_2$Me

The orphan nuclear receptor NR4A1 is overexpressed in ER-positive and ER-negative breast cancer cells (Muscat et al. 2013) and the role of this receptor in regulating breast cancer cell growth and survival was investigated by RNA interference (RNAi) in ER-positive (MCF-7), ER-negative (MDA-MB-231) and erbB2 (SKBR3) overexpressing breast cancer cell lines. Cells were transfected with two different oligonucleotides against NR4A1 (siNR4A1-1/siNR4A1-2) and this resulted in ≥50% growth inhibition in MCF-7 and SKBR3 cells and 35% inhibition of MDA-MB-231 cell proliferation (Fig. 1B). The C-DIM compounds with a *p*-carboxymethylpenyl group (DIM-C-pPhCO$_2$Me) and cyano substitutent (DIM-C-pPhCN) have been identified as an NR4A1 antagonists (Lee et al. 2014b) and Figure 1C and Supplemental Figure S1A demonstrate that the former compound significantly inhibits growth of MCF-7, MDA-MB-231 cells after treatment for 24 and 48 hr. IC_{50} values were 20, 19 and 19 μM after treatment of MCF-7, MDA-MB-231 and SKBR3 cells, respectively, for 24 hr and 13, 19

and 12 μM after treatment for 48 hr. Moreover, in an orthotopic model for breast cancer in athymic nude mice using MDA-MB-231 cells treatment with 50 mg/kg/d and weight compared to the corn oil controls (Fig. 1D). In contrast, after knockdown of NR4A1 in these cells, treatment with DIM-C-pPhCO_2Me resulted in only minimal growth inhibition confirming a role for NR4A1 in mediating the growth inhibitory effects of DIM-C-pPhCO_2Me (Fig. 1E).

2. siNR4A1 and DIM-C-pPhCO_2Me induce apoptosis in breast cancer cells

NR4A1 also regulates pro-survival genes and pathways in pancreatic and lung cancer cells, and results in Figure 2A show that transfection of breast cancer cells with siNR4A1 induced cleavage of caspase 8 and caspase 7 and also PARP cleavage. Moreover, siNR4A1 also induced annexin V staining MCF-7 (Fig. 2B), MDA-MB-231 (Fig. 2C), and SKBR3 (Fig. 2D) cells confirming that NR4A1 regulated anti-apoptotic pathways in these cell lines. Treatment of the cells with DIM-C-pPhCO_2Me for 24 hr also induced cleavage (activation) of caspases 7 and 8 and PARP (Fig. 3A) and similar results were observed for the *p*-cyanophenyl compound (DIM-C-pPhCN) which is an NR4A1 ligand and antagonist in colon cancer cells (Lee et al. 2014b) (Suppl. Fig. S1B). DIM-C-pPhCO_2Me also enhanced annexin V staining in MCF-7 (Fig. 3B), MDA-MB-231 (Fig. 3C), and SKBR3 (Fig. 3D) cell lines. Thus, both NR4A1 knockdown and NR4A1 antagonists decreased breast cancer cell growth and induced apoptosis.

3. siNR4A1 and DIM-C-pPhCO_2Me activate growth inhibitory pro-apoptotic pathways/genes in breast cancer cells

mTOR activation in lung and colon cancer cells is dependent on NR4A1-p53 interactions that inactivate p53, and siNR4A1 or NR4A1 antagonists activate p53 which induces expression of sestrin 2, resulting in phosphorylation of AMPKα and inhibition of mTOR (Lee et al. 2012; Lee et al. 2014b). Knockdown of NR4A1 in p53-wild-type MCF-7 cells resulted in the induction of sestrin 2 and phosphorylation of AMPKα and this was accompanied by decreased activation (phosphorylation) of the mTOR downstream gene products p70S6K and S6RP (Fig. 4A). MCF-7 cells were also treated with the NR4A1 antagonist DIM-C-pPhCO_2Me (Fig. 4B) and results were similar to that observed after transfection with siNR4A1. The NR4A1 antagonist DIM-C-pPhCN also inhibited the mTOR pathway (Suppl. Fig. S1C). Previous studies also show that NR4A1 regulates expression of growth promoting and pro-survival (e.g. survivin, bcl-2 and EGFR) genes through interactions with Sp1 bound to their corresponding proximal GC-rich *cis*-promoter elements (Lee et al. 2010; Lee et al. 2012; Lee et al. 2014b). Figure 4C shows that after knockdown of NR4A1 (siNR4A1) in MCF-7, MDA-MB-231 and SKBR3 cells, there was a significant decrease in expression of several Sp1-regulated genes including EGFR, survivin and bcl-2; however, Sp1 protein levels were unchanged. Similar results were observed in the same cell lines after treatment with the NR4A1 antagonists DIM-C-pPhCO_2Me (Fig. 4D) and DIM-C-pPhCN (Suppl. Fig. S1D). These observations are consistent with previous reports in pancreatic, colon and lung cancer cells where NR4A1 regulates expression of survivin and other Sp1-regulated genes (Lee et al. 2010; Lee et al. 2012; Lee et al. 2014b).

It was recently reported that NR4A1 regulates expression of genes such as IDH1 and TXNDC5 that maintain high levels of reducing equivalents and minimize ROS-

mediated cellular stress (Lee et al. 2014a; Lee et al. 2014b). Knockdown of NR4A1 by RNAi induced ROS by 2- to 4-fold in MCF-7, MDA-MB-231 and SKBR3 cells and this was accompanied by decreased expression of both TXNDC5 and IDH1 in these cell lines (Fig. 5B). Moreover, after transfection with siNR4A1, we also observed enhanced markers of ER stress including increased phosphorylation of PERK and increased expression of ATF-4, CHOP and spliced XBP-1 (XBP-1s) in the breast cancer cell lines (Fig. 5C). Treatment of MCF-7, MDA-MB-231 and SKBR3 cells with the NR4A1 antagonist DIM-C-pPhCO_2Me also increased ROS after 12 and 24 hr (Figs. 6A-6C) and this was also accompanied by decreased expression of TXNDC5 and IDH1 and induction of markers of ER stress (p-PERK, ATF4, CHOP and XBP-1s) as previously observed in pancreatic cancer cells (Lee et al. 2014a). We also observed that the NR4A1 antagonist DIM-C-pPhCN decreased expression of IDH1 and TXNDC5 in breast cancer cells (Suppl. Fig. S1E). In addition, Western blot analysis of tumor lysate from control and DIM-C-pPhCO_2Me-treated mice confirmed that DIM-C-pPhCO_2Me significantly induced PARP cleavage, decreased expression of Sp-regulated survivin, EGFR and bcl2 gene products and also decreased levels of TXNDC5 and IDH1 (Suppl. Figs. S2A and S2B). These results are consistent with the *in vitro* effects of DIM-C-pPhCO_2Me (and siNR4A1) in breast cancer cells and demonstrates that C-DIM/NR4A1 antagonists inhibit common NR4A1-mediated pro-oncogenic pathways in breast, pancreatic, colon and lung cancer cell lines.

DISCUSSION

NR4A1 exhibits pro-oncogenic activity in cancer cell lines derived from solid tumors and is overexpressed in tumors from lung, pancreatic and colon cancer patients (Chintharlapalli et al. 2005; Cho et al. 2010; Lee et al. 2010; Lee et al. 2012; Lee et al. 2014a; Wu et al. 2011). Moreover, in lung cancer patients, high expression of NR4A1 is a prognostic indicator for decreased survival (Lee et al. 2012). Similar results were recently reported for the expression and prognostic activity of NR4A1 in breast cancer patients, and it was also observed that NR4A1 was one of only a few nuclear receptors overexpressed in patients with both ER-positive and ER-negative breast tumors (Muscat et al. 2013; Zhou et al. 2014). Since an early report indicated that NR4A1 is more highly expressed in early vs. late stage more aggressive breast tumors and exhibited some tumor suppressor-like activity (Alexopoulou et al. 2010), we investigated the function of NR4A1 in three different breast cancer cell lines by RNA interference and treatment with NR4A1 antagonists. We have recently demonstrated that several C-DIMs including the *para*-hydroxy, carbomethoxy, cyano and bromophenyl analogs directly bind the ligand binding domain of NR4A1 and exhibit NR4A1 antagonist activity in colon cancer cells (Lee et al. 2014b). The *p*-carbomethyoxyphenyl analog (DIM-C-pPhCO_2Me) and to a lesser extend DIM-C-pPhCN were used as prototypical C-DIMs/NR4A1 antagonists for investigating the anticancer activities of NR4A1 antagonists in breast cancer cells with a focus on inhibition of 3 previously identified pro-oncogenic NR4A1-regulated pathways (Fig. 1A).

Initial studies investigated the role of NR4A1 in the growth of three prototypical breast cancer cell lines (ER-positive MCF-7, erbB2 overexpressing SKBR3, and triple

negative MDA-MB-231 cells), and knockdown of NR4A1 using two different oligonucleotides significantly decreased proliferation of all three breast cancer cell lines and similar growth inhibitory effects were observed for DIM-C-pPhCO_2Me (Figs. 1B and 1C). Moreover, after knockdown of NR4A1 in MCF-7, MDA-MB-231 and SKBR3 cells, treatment with DIM-C-pPhCO_2Me had minimal effects (Fig. 1D), suggesting that the growth inhibitory effects of DIM-C-pPhCO_2Me were primarily NR4A1-dependent. The effects of NR4A1 knockdown or treatment with DIM-C-pPhCO_2Me on several markers of apoptosis, including cleavage of caspases 7 and 8 and PARP and induction of annexin V staining, were also determined in the breast cancer cell lines (Figs. 2 and 3). These results demonstrate that NR4A1 regulates pathways that contribute to the growth and survival of breast cancer cells and this parallels the functions previously observed for this receptor in pancreatic, lung and colon cancer cells (Lee et al. 2010; Lee et al. 2012; Lee et al. 2014a; Lee et al. 2014b).

NR4A1 binds and inactivates p53 (Zhao, et al. 2006), and knockdown of NR4A1 or treatment of p53 wild-type lung cancer cells with an NR4A1 antagonist or transfection with siNR4A1 results in activation of p53 and induction of sestrin 2 which activates AMPKα and inhibits the mTOR pathway (Lee et al. 2012). mTOR pathway inhibitors have been extensively developed for cancer chemotherapy (Baselga, et al. 2012; Ciruelos Gil 2014), and C-DIM/NR4A1 antagonists represent a new class of mTOR inhibitors which block NR4A1-regulated mTOR activation in cancer cells expressing wild-type p53 (Lee et al. 2012). Results illustrated in Figures 4A and 4B show that both siNR4A1 and DIM-C-pPhCO_2Me inhibited mTOR pathway in MCF-7 breast cancer cells that express wild-type p53. In p53 wild-type lung cancer cells, siNR4A1 and NR4A1

antagonists also induced sestrin 2 which activates AMPKα and inhibits mTOR, whereas this is not observed in lung cancer cells expressing mutant p53 (Lee et al. 2012). Interestingly, DIM-C-pPhCO_2Me and siNR4A1 also induced sestrin 2 and inhibited mTOR in p53 mutant SKBR3 and MDA-MB-231 cells (data not shown) and the mechanisms of this response are currently being investigated.

Like other nuclear receptors, NR4A1 interacts with the Sp1 transcription factor bound to GC-rich sites to activate survivin and other anti-apoptotic/growth promoting genes, and siNR4A1 or treatment with a NR4A1 antagonist decreases expression of these genes (Liu and Simpson 1999; Lu, et al. 2000; Pipaon, et al. 1999; Shimada, et al. 2001; Sugawara, et al. 2002; Suzuki, et al. 1999). Figures 4C and 4D show that siNR4A1 or treatment with DIM-C-pPhCO_2Me decreased expression of survivin, bcl2 and EGFR in MDA-MB-231, MCF-7 and SKBR3 cells; however, Sp protein levels were unchanged. Molecular analysis of NR4A1-dependent regulation of survivin showed that in pancreatic cancer cells, NR4A1 and p300 cooperatively activated survivin expression by interacting with Sp1 bound to the proximal GC-rich region of the survivin promoter (Lee et al. 2010). Regulation of growth-promoting and survival genes which contain GC-rich promoters by NR4A1 is consistent with the growth inhibitory and apoptotic effects of siNR4A1 and C-DIM/NR4A1 antagonists on breast cancer cells and tumors and is comparable to that observed in lung, colon and pancreatic cancer cell lines (Lee et al. 2010; Lee et al. 2012; Lee et al. 2014a; Lee et al. 2014b).

A recent study showed that NR4A1 maintains low levels of oxidative and endoplasmic reticulum (ER) stress in pancreatic cancer cells by regulating expression of TXNDC5 and IDH1 which maintain cellular levels of reducing equivalents (Lee et al.

2014a). DIM-C-pPhCO$_2$Me or siNR4A1 decreased expression of TXNDC5 and IDH1 in breast tumors (*in vivo*) (Suppl. Fig. S2) and in MCF-7, MDA-MB-231 and SKBR3 cells and this was accompanied by increased levels of ROS and induction of markers of ER stress (Figs. 5 and 6). These results are consistent with previous studies in pancreatic cancer cells, and there is also emerging evidence that both TXNDC5 and IDH1 are overexpressed in breast cancer cells and tumors (Chang, et al. 2013; Xu, et al. 2010) and their expression and functions in breast cancer cells are currently being investigated.

In summary, results of this study are consistent with a pro-oncogenic role for NR4A1 in breast cancer as an important regulator of cell growth and survival, and NR4A1-regulated pro-oncogenic pathways and genes are similar to those observed in pancreatic, lung and colon cancer cells and tumors (Lee et al. 2010; Lee et al. 2012; Lee et al. 2014a; Lee et al. 2014b). This study also demonstrated the effectiveness of the NR4A1 antagonists DIM-C-pPhCO$_2$Me and DIM-C-pPhCN as inhibitors of breast cancer cell and tumor growth and survival, and current structure-activity studies are focused on identifying the most effective C-DIM/NR4A1 antagonists for future clinical applications in breast cancer chemotherapy, including the inhibition of breast invasion (Zhou et al. 2014) through inhibition of nuclear NR4A1 by receptor antagonists.

Disclosures: There are no disclosures to declare.

Funding: Funding was provided by DOD-CDRMP (BC103116) (M.S.), NIEHS (P30-ES023512) (S.S.), Texas AgriLife Research, and the Sid Kyle Endowment (S.S.).

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FIGURE CAPTIONS

Figure 1. NR4A1-regulated pathways and effects of NR4A1 knockdown on breast cancer cell proliferation. (A) NR4A1-regulated pathways/genes that can be targeted by C-DIM/NR4A1 antagonists. (B) Cells were transfected with two siNR4A1 oligonucleotides (1 and 2) and cell numbers were determined after 72 hr. (C) Cells were treated with different concentrations of DIM-C-pPhCO$_2$Me for 24 hr and the number of cells were then determined. (D) Athymic nude mice bearing MDA-MB-231 cells (orthotopic) were administered corn oil (control), DIM-C-pPhCO$_2$Me or DIM-C-pPhCN (50 mg/kg d) by oral gavage for 28 days, and effects on tumor growth and weight were determined (* significantly decreased; $p<0.01$). (E) Cells were transfected with siCtl (non-specific oligonucleotide) or siNR4A1 and then treated with 20 μM DIM-C-pPhCO$_2$Me for 24 hr and the number of cells were then counted. Results (B – D) are means $\pm$ SE for at least 3 separate determinations for each treatment group. Significant ($p < 0.05$) growth inhibition is indicated (*) and a significant decrease in the growth inhibitory effects of DIM-C-pPhCO$_2$Me after NR4A1 knockdown is also indicated (**).

Figure 2. siNR4A1 and DIM-C-pPhCO$_2$Me induce apoptosis in breast cancer cells. (A) Cells were transfected with siNR4A1 and whole cell lysates were analyzed by western blots. Cells were transfected with siNR4A1 (2 oligonucleotides) and effects on Annexin V staining in MCF-7 (B), MDA-MB-231 (C) and SKBR3 (D) cells were determined and quantitated. Results (B – D) are means $\pm$ SE for at least 3 separate determinations and significant ($p < 0.05$) induction of Annexin V staining is indicated (*).

Figure 3. DIM-C-pPhCO$_2$Me induces apoptosis in breast cancer cells. (A) Cells were treated with DIM-C-pPhCO$_2$Me for 24 hr and whole cell lysates were analyzed by western blots. MCF-7 (B), MDA-MB-231 (C) and SKBR3 (D) cells were treated with DIM-C-pPhCO$_2$Me for 24 hr and Annexin V staining was determined and quantitated. Quantitative results are means ± SE for 3 separate determinations and significant ($p < 0.05$) induction of Annexin V is indicated (*).

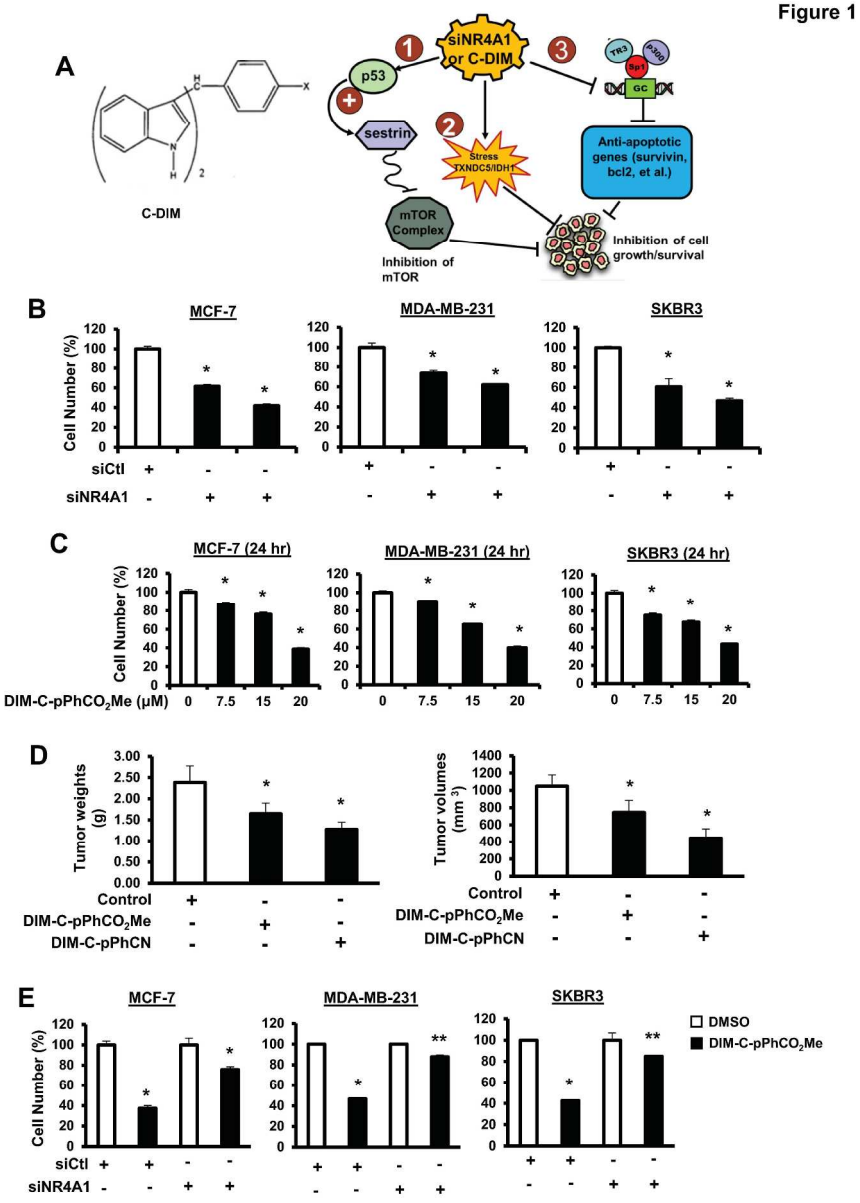
Figure 4. NR4A1 knockdown inhibits mTOR and selected genes with GC-rich promoters. MCF-7 cells were transfected with siNR4A1 (A) or treated with DIM-C-pPhCO$_2$Me (B) and whole cell lysates were analyzed by Western blots for mTOR pathway gene products. Cells were transfected with siNR4A1 (C) or treated with DIM-C-pPhCO$_2$Me (D) and whole cell lysates were analyzed by western blots for selected genes with GC-rich promoters.

Figure 5. NR4A1 knockdown induces ROS and cellular stress. (A) Cells were transfected with siNR4A1 or siCtl and ROS was determined. (B) Cells were transfected with siNR4A1 and whole cell lysates were analyzed by western blots for IDH1 or TXNDC5 and ER stress gene products. Results in (A) are means ± SE for 3 separate determinations and significant ($p < 0.05$) induction of ROS is indicated (*).

Figure 6. DIM-C-pPhCO$_2$Me induces ROS and stress. MCF-7 (A), MDA-MB-231 (B) and SKBR3 (C) cells were treated with DIM-C-pPhCO$_2$Me and ROS was determined after 12 or 24 hr. (D) Cells were treated with DIM-C-pPhCO$_2$Me and whole cell lysates

were analyzed by western blots for expression of TXNDC5, IDH1 and stress gene products. Results (A) are expressed as means ± SE for 3 separate determinations and significant ($p < 0.05$) induction of ROS is indicated (*).

Figure 1



NR4A1-regulated pathways and effects of NR4A1 knockdown on breast cancer cell proliferation.
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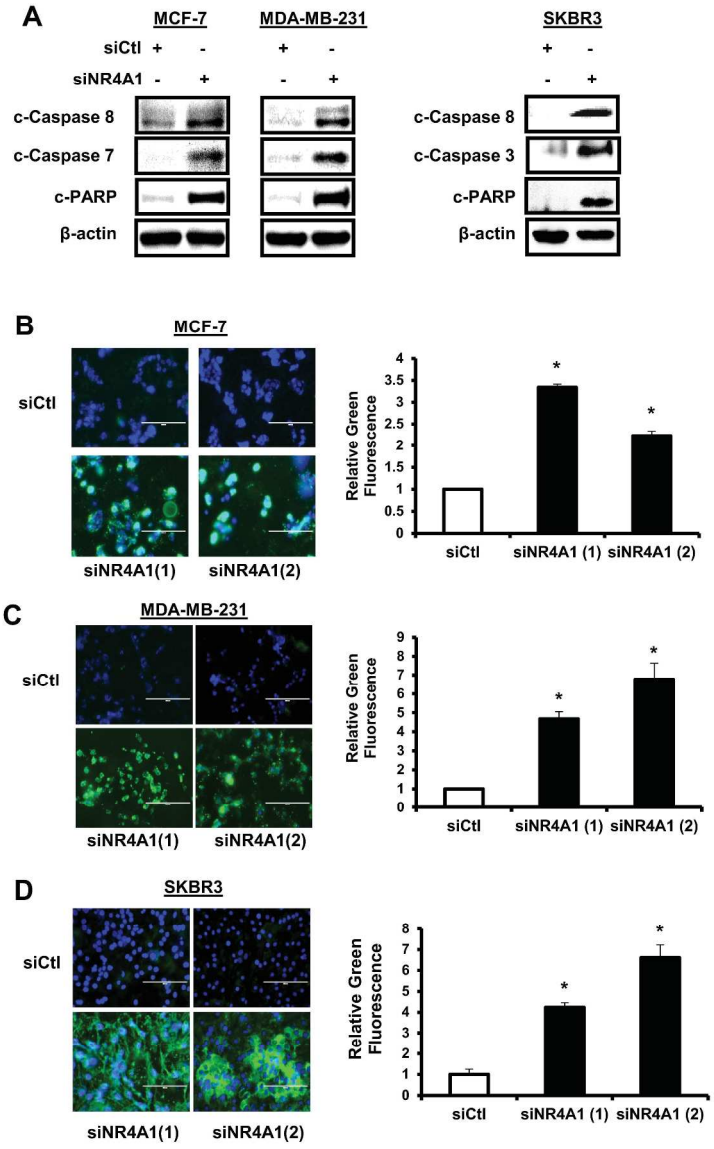
Figure 2



siNR4A1 and DIM-C-pPhCO2Me induce apoptosis in breast cancer cells.
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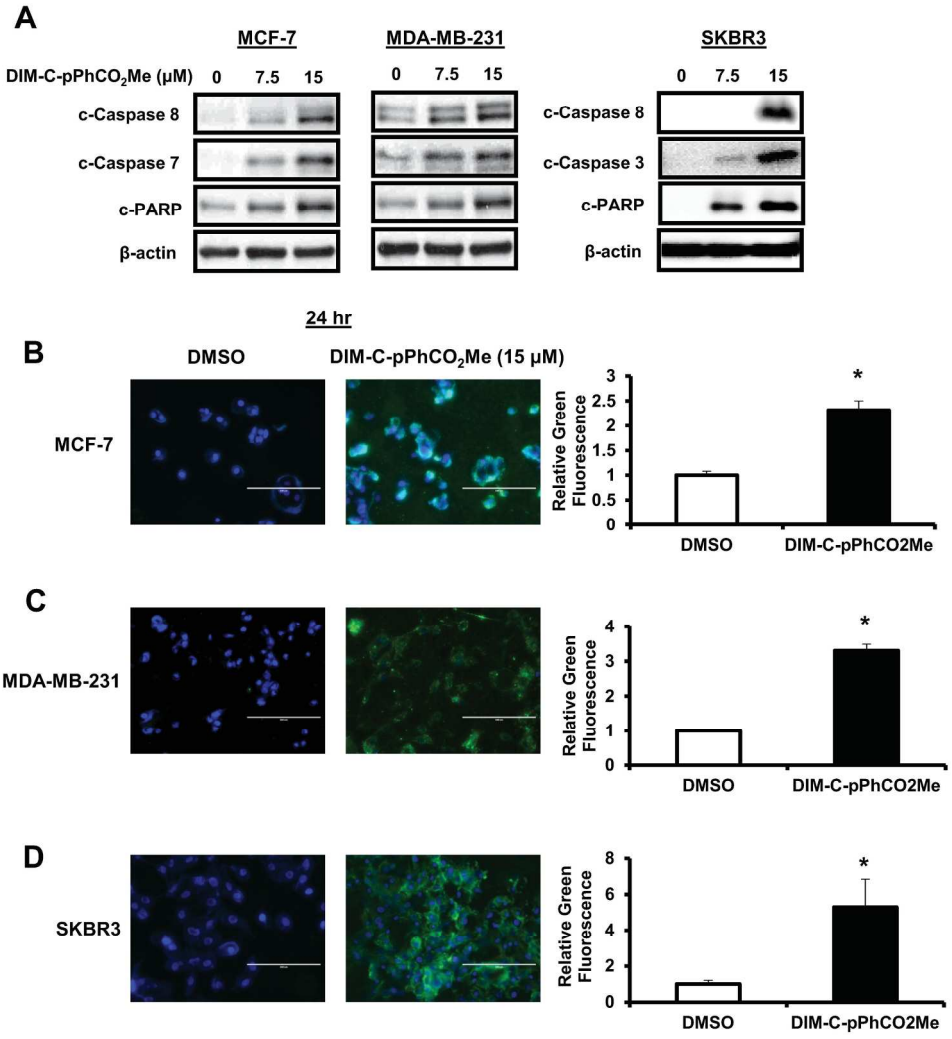
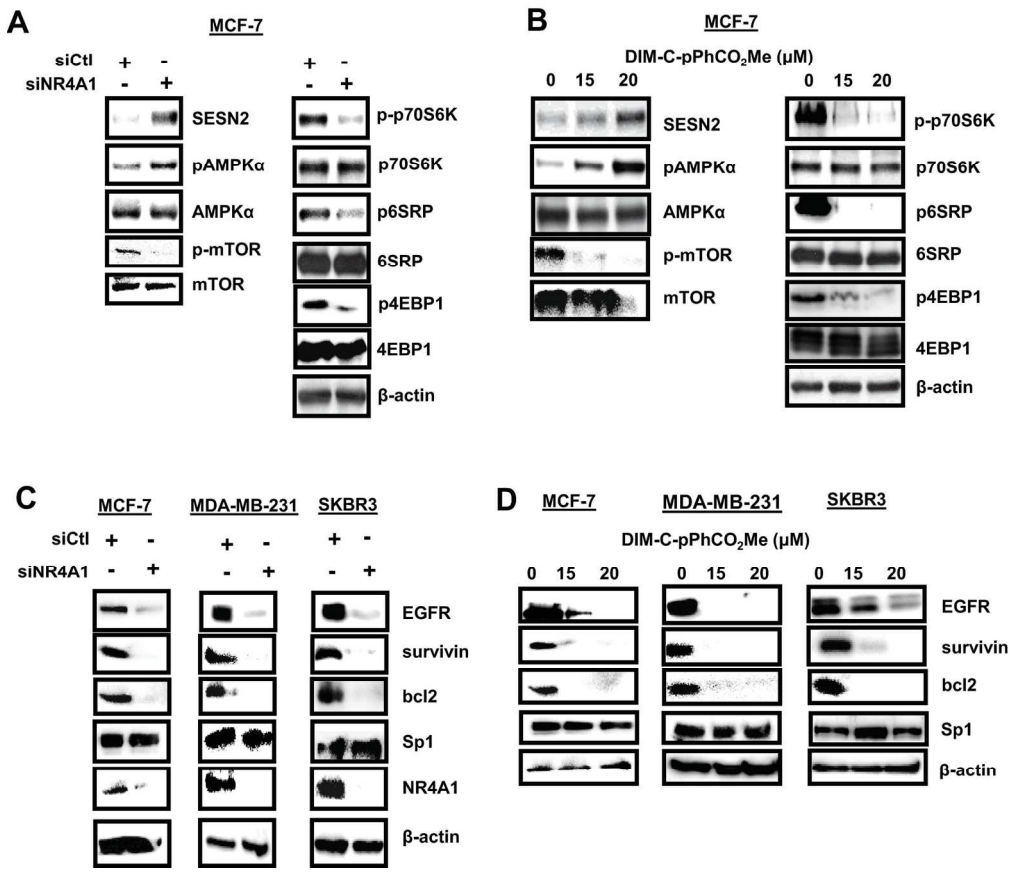
Figure 3



DIM-C-pPhCO2Me induces apoptosis in breast cancer cells.
208x237mm (300 x 300 DPI)

Figure 4



NR4A1 knockdown inhibits mTOR and selected genes with GC-rich promoters.
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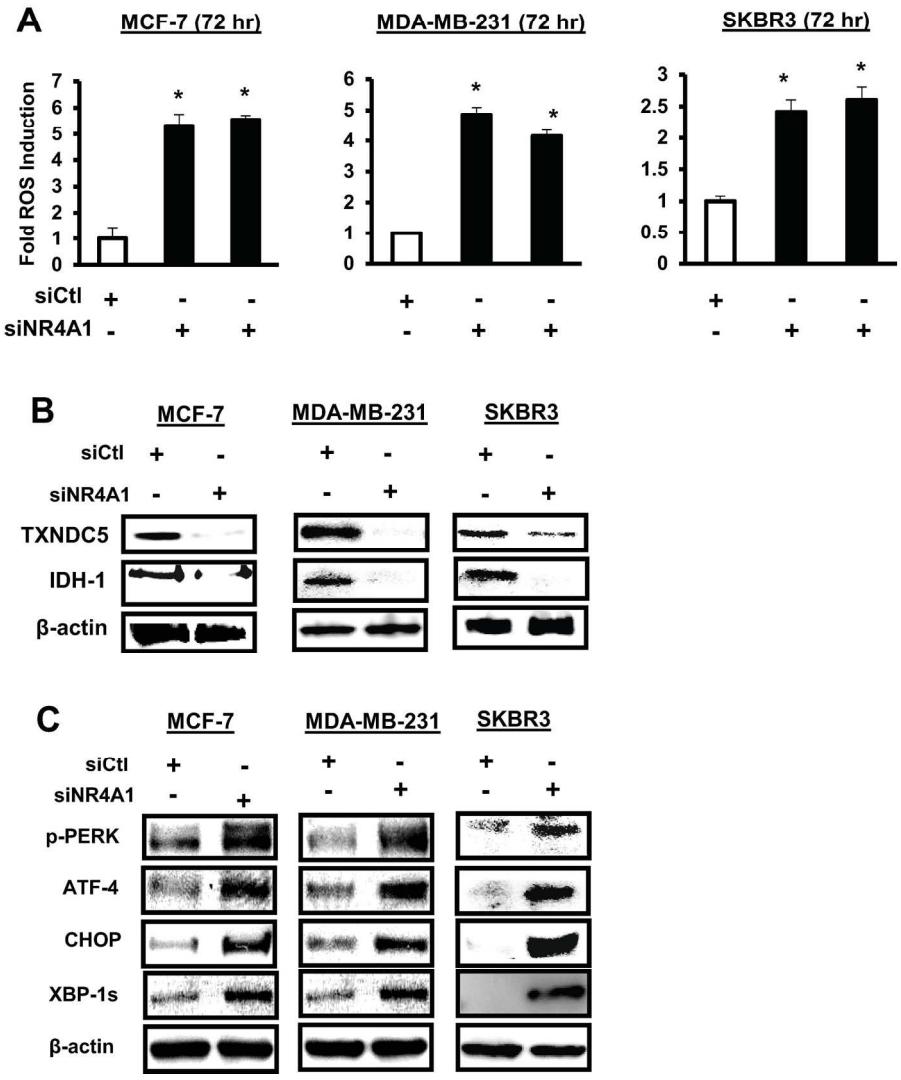
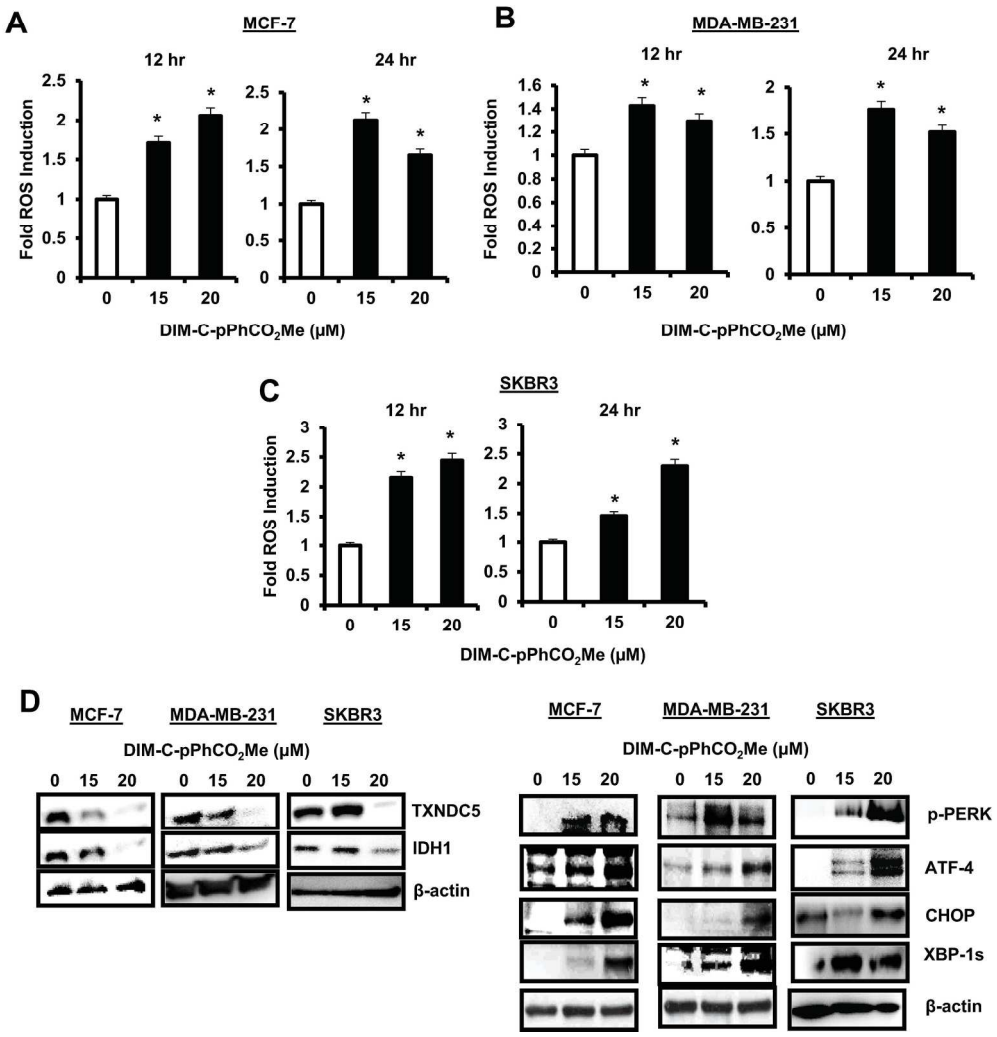
Figure 5



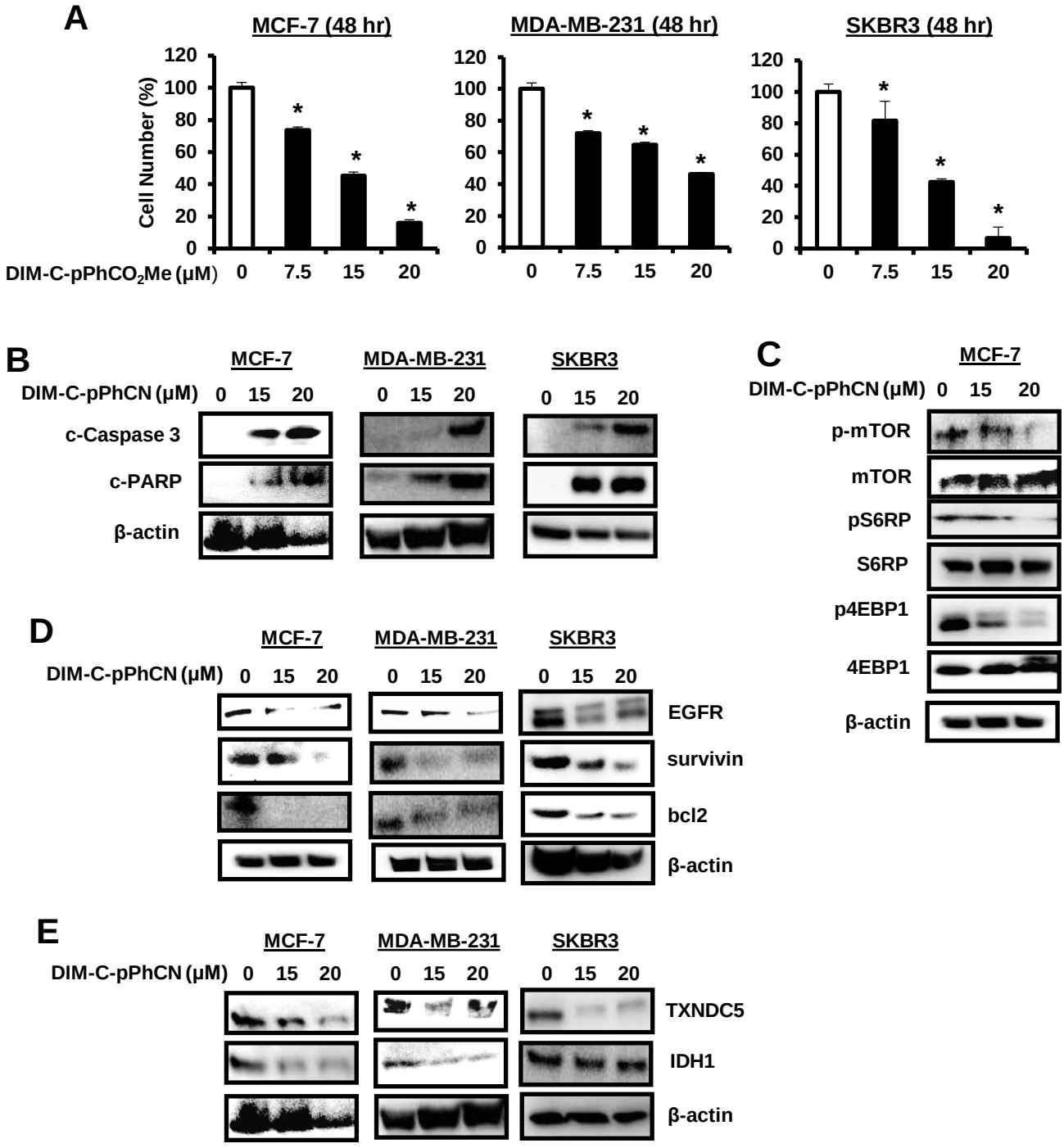
NR4A1 knockdown induces ROS and cellular stress.
185x213mm (300 x 300 DPI)

Figure 6



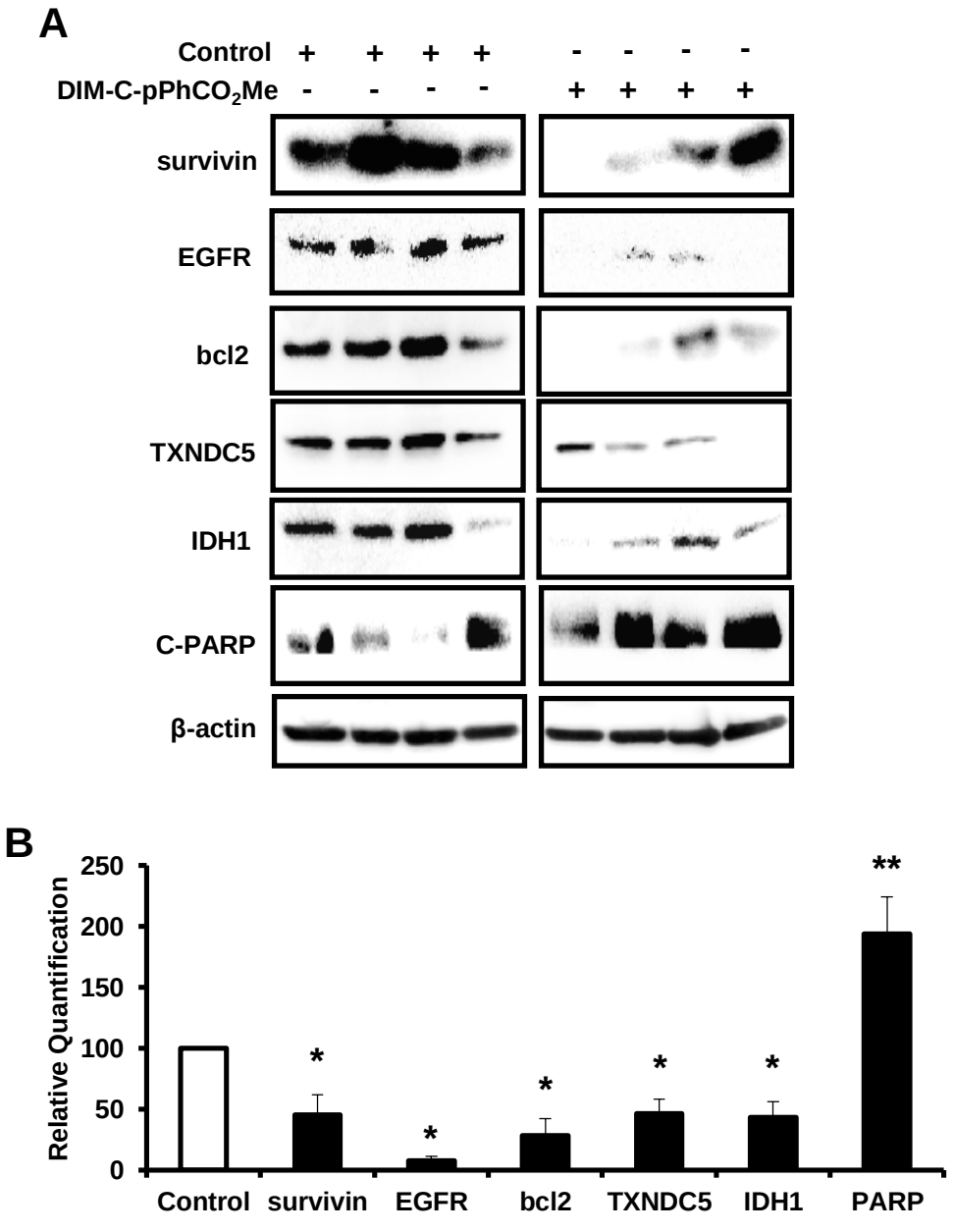
DIM-C-pPhCO2Me induces ROS and stress.
203x225mm (300 x 300 DPI)

Supplemental Figure S1



Supplemental Figure S1. Effects of NR4A1 antagonists on breast cancer cells. (A) Cells were treated with DIM-C-pPhCO$_2$Me for 48 hr and cell numbers were determined. Cells were treated with DIM-C-pPhCN for 24 hr and whole cell lysates were analyzed by Western blots for markers of apoptosis (B), mTOR signaling (C), expression of genes with GC-rich promoters (D), and stress markers (E).

Supplemental Figure S2



Supplemental Figure 2. Effects of NR4A1 antagonists on selected gene product expression in tumors. (A) Orthotopic tumors in athymic nude mice bearing MDA-MB-231 cells were treated with DIM-C-pPhCO$_2$Me (50 mg/kg/d) or vehicle (control) for 28 days. (A) Lysates from tumors from control and DIM-C-pPhCO$_2$Me-treated mice were analyzed by western blots and (B) bands were quantitated and normalized relative to β-actin.